PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
G01N 33/53, 33/573, A61K 31/70, 38/16, 48/00

A1

(11) International Publication Number: WO 95/21380

(43) International Publication Date: 10 August 1995 (10.08.95)

(21) International Application Number: PCT/US95/01375

(22) International Filing Date: 2 February 1995 (02.02.95)

(30) Priority Data:

PM 3650	2 February 1994 (02.02.94)	AU
PM 6638	5 July 1994 (05.07.94)	AU
PM 7779	31 August 1994 (31.08.94)	AU

(81) Designated States: AU, CA, CN, FI, JP, KR, NO, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

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(54) Title: ASSAY FOR SCREENING OF RECEPTOR ANTAGONISTS

(57) Abstract

The invention provides a method of identifying a compound having the ability to block the low-affinity gastrin-cholecystokinin type C receptor, comprising the step of measuring the ability of said compound to block the binding to gastrin-binding protein of a compound selected from the group consisting of a gastrin-related peptide, a cholecystokinin-related peptide, an antagonist of gastrin or of cholecystokinin, an acyl CoA, an enoyl CoA, an antibody to gastrin, and an antibody to cholecystokinin. Several ways in which the method of the invention can be carried out are described. Thus, the invention provides a general screening method for identifying antagonists of the gastrin-binding protein interaction, which are useful for treatment of diseases involving rapidly proliferating cells, and for controlling gastric acid secretion. Compositions and methods of treatment are also claimed.

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ASSAY FOR SCREENING OF RECEPTOR ANTAGONISTS

This invention relates to a method for screening of putative antagonists of binding of gastrin to its receptor, in particular the low-affinity gastrin-cholecystokinin C receptor, more particularly the gastrin-binding protein (GBP), and also of inhibitors of the enzyme activities associated with the GBP.

Compounds having the ability to block binding of gastrin to GBP or to block the enzyme activities of the GBP are useful in control of cellular proliferation, especially in treatment of neoplastic disease, and are also useful in the control of acid production in the stomach.

BACKGROUND OF THE INVENTION

The hormone gastrin has been known for over 80 years to be a stimulant of acid secretion by the parietal cells of the stomach. Gastrin is a small polypeptide hormone which participates in a feedback system for regulating the environment in the lumen of the alimentary tract, and is thought to affect other target organs, including the brain and pancreas. More recently, it has been recognised that gastrin also acts as a growth factor for the gastric mucosa. Both of these functions are presumably mediated by the binding of gastrin to specific receptors on the target cells in the gastrointestinal mucosa. A variety of methods for detection of such receptors in tissue, cell, and membrane preparations from various tissues has been demonstrated.

Gastrin/CCK-C receptors have been identified on cell lines of a variety of tumour cell lines of epithelial and non-epithelial origin, and on several cell lines of lymphocytic origin. In particular, significant levels of gastrin/CCK-C receptors have been observed with cell lines established from carcinomas of the stomach, colon, breast, and vulva, and from malignant melanoma: in addition, lower levels of binding were observed with T- and B-cell lymphoma

cell lines, while still smaller levels of binding were observed with promyelocytic and myeloid leukaemia cell lines (3).

Three classes of receptors for gastrin and for the related hormone cholecystokinin (CCK) have been described. The CCK-A receptor on the pancreatic acinar cell, and the gastrin/CCK-B receptor in the gastric mucosa and brain, both belong to the family of receptors with 7 transmembrane segments. These two receptors can be readily distinguished with the CCK-A receptor-selective antagonist L364,718, and the gastrin/CCK-B receptor-selective antagonist L365,260. Characteristics of the three types of receptors are summarised in Table 1 below.

Evidence that a gastrin-like peptide acts as an autocrine growth factor in colorectal carcinoma has been accumulating steadily. Gastrin mRNA has been demonstrated in both normal and neoplastic colorectal mucosa by Northern blotting, and in colon carcinoma cell lines by the polymerase chain reaction. An increase in progastrin production in colorectal tumour tissue compared with normal mucosa has been demonstrated in tumour sections by immunohistochemistry, and in tissue extracts by radioimmunoassay. Progastrin, but not mature amidated gastrin, was also produced by all (5/5) colonic carcinoma cell lines tested. Exogenous $gastrin_{17}$ or its derivatives enhanced the growth of xenografts of 60% of colon carcinomas tested. However, many colon carcinoma cell lines do not increase either DNA or protein synthesis *in vitro* in response to exogenous gastrin, perhaps because they are already maximally stimulated by an autocrine gastrin-like peptide. In some cases previously unresponsive cells have been rendered responsive by synchronisation.

The most convincing evidence for an autocrine role for a gastrin-like peptide has been provided by studies with gastrin/cholecystokinin (CCK) receptor agonists and antagonists. The antagonist proglumide, which

does not discriminate clearly between the three known classes of gastrin/CCK receptors, increases the survival time of mice bearing tumours derived from the mouse colon carcinoma line MC26 (1). Proglumide also reverses the increase in tumour volume observed in tumour-bearing mice after treatment with pentagastrin. Proliferation of 6 colon carcinoma cell lines *in vitro* is inhibited by the non-selective antagonists proglumide and benzotript, with IC_{50} values in the mM range (2). Furthermore, in the case of the HCT 116 cell line proliferation is also inhibited by an anti-gastrin antiserum (2). The high concentrations of gastrin required to reverse inhibition by both antagonists and antibodies suggest that neither pancreatic CCK-A nor gastric gastrin/CCK-B receptors are involved, but are consistent with the involvement of the low affinity gastrin/CCK-C receptor which we have identified on the surface of several gastric and colonic carcinoma cell lines (2). The failure of the selective antagonists L364,718 and L365,260 to inhibit proliferation of HCT 116 cells at concentrations as high as 1 μM confirms that neither CCK-A nor gastrin/CCK-B receptors participate in the autocrine loop (4).

We have identified a gastrin-binding protein (GBP) in detergent extracts of porcine gastric mucosal membranes. The GBP has a molecular weight of 78kDa, and does not appear to have any other disulphide bonded sub-units (5). Purification of the porcine GBP by a combination of lectin and ion exchange chromatography followed by preparative gel electrophoresis has permitted determination of N-terminal and internal amino acid sequences of the naturally occurring protein (Baldwin et al: Int. J. Biochem 26 529-538). These sequences have been used to generate probes which have enabled cloning of the cDNA encoding the porcine GBP from both c-DNA and genomic libraries.

The sequence of porcine GBP, deduced from the cDNA sequence, is related to those of enzymes having enoyl-

CoA hydratase activity and to enzymes having 3-hydroxy-acyl-CoA dehydrogenase activity. These enzymes are involved in two consecutive steps in the synthesis and degradation of fatty acids during metabolism located in the α-subunit of a mitochondrial trifunctional protein (MTP). The N-terminal half of the GBP is related to the enoyl-CoA hydratase family, while the C-terminal half is related to the 3-hydroxy-acyl-CoA dehydrogenase family (6). In particular, the sequence of the porcine GBP is closely related to the sequence of the α-subunit of a rat mitochondrial trifunctional protein (MTP), which catalyses both activities. We have found that the sequence of mature human GBP is 90% identical to that of the porcine GBP (Zhang and Baldwin, submitted for publication).

To investigate the possibility that the GBP might be involved in the autocrine effects of gastrin-like peptides on colorectal carcinoma cells, we have now examined the effect of agonists and antagonists on the interaction between the GBP and gastrin. Since colon carcinoma cell lines synthesise progastrin, but fail to process the prohormone to mature C-terminally amidated $gastrin_{17}$, a candidate receptor for autocrine gastrin should not require an amidated C-terminus for binding. We have therefore also examined the structural requirements for binding of C-terminally extended gastrins to the GBP.

We have now found that covalent cross-linking of ^{125}I-[Nle^{15}]-$gastrin_{2,17}$ to the 78 kDa GBP is inhibited by crotonyl CoA and by acetoacetyl CoA. The finding that enoyl CoA and acyl CoA derivatives inhibit binding of gastrin to GBP is surprising. According to the autocrine hypothesis, the reverse effect would be expected. Gastrin, CCK, and their analogues also inhibit cross-linking, and the spectrum of analogue affinities correlates well with the values previously reported for binding to the gastrin/CCK-C receptor, but poorly with the values reported for binding to either the CCK-A or gastrin/CCK-B receptors. The good correlation observed between the IC_{50} values for

antagonists in the GBP cross-linking assay and the IC_{50} values for inhibition of either binding of gastrin to colon carcinoma cells or of cell proliferation suggests that the GBP is the target for the inhibitory effects of gastrin/CCK receptor antagonists on cell growth. Cross-linking is also inhibited by proglumide and benzotript, but no inhibition is seen with either the CCK-A receptor-selective antagonist L364,718 or the gastrin/CCK-B receptor-selective antagonist L365,260. The affinities of antagonists for the GBP correlate well with their affinities for the gastrin/CCK-C receptor, and with their potencies for inhibition of colon carcinoma cell growth. In addition to gastrin/CCK receptor antagonists, we have found that other classes of compounds inhibit the cross-linking. For example, non-steroidal anti-inflammatory drugs inhibit not only the cross-linking, but also proliferation of a human colon carcinoma cell line.

To investigate further the postulated autocrine loop for gastrin we have transfected two colon cell lines with a plasmid encoding a gastrin cDNA in the antisense orientation. We have observed inhibition of cell proliferation, providing clear evidence for the presence of an autocrine loop. Moreover the failure to reverse inhibition with exogenous $gastrin_{17}$ suggests that the loop in these cells is intracellular.

We conclude that the 78 kDa gastrin-binding protein is

(i) a member of the hydratase/dehydrogenase family of fatty acid oxidation enzymes;

(ii) the gastrin/CCK-C receptor;

(iii) the target for the anti-proliferative action of gastrin/CCK receptor antagonists and compounds such as non-steroidal anti-inflammatory drugs;

(iv) a potential target for the autocrine effects of progastrin; and

(v) a target for anti-sense gastrin RNA regulation of proliferation of colon carcinoma cells.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides a method of identifying a compound having the ability to block the low-affinity gastrin-cholecystokinin type C receptor, comprising the step of measuring the ability of said compound to block the binding to gastrin-binding protein of a compound selected from the group consisting of a gastrin-related peptide, a cholecystokinin-related peptide, an antagonist of gastrin or of cholecystokinin, an acyl CoA, an enoyl CoA, an antibody to gastrin, and an antibody to cholecystokinin.

The binding may be reversible or irreversible, but is preferably irreversible.

In a preferred embodiment, inhibition of irreversible binding is assessed by measuring the ability of the compound to block cross-linking of a gastrin analogue to gastrin binding protein.

Cross-linking may be effected by an agent which is homobifunctional, heterobifunctional, or photoreactive. The person skilled in the art will be aware of a number of suitable reagents. Examples of commercially-available homobifunctional cross-linking reagents include disuccinimidyl suberate, disulfosuccinimidyl suberate, and ethylene glycol bis-(succinimidyl)-succinate. Heterobi-functional cross-linking reagents include succinimidyl-4-(p-maleimidophenyl) butyrate, which has been found to be equally effective in cross-linking the gastrin peptide to the gastrin binding protein. Suitable photoreactive reagents include N-hydroxysuccinimidyl-4-azidobenzoate.

The gastrin peptide is labelled with a detectable marker, such as a radioactive label, a fluorescent label, or biotin. For example, the gastrin peptide may be iodinated, or may be synthesised using one or more amino

acids comprising a radioactive marker such as ^{14}C or ^{3}H.

Native gastrin, gastrin$_{17}$, contains no free amino groups, and is therefore incapable of reacting with the cross-linking reagent. The minimum gastrin sequence required for binding to the gastrin/CCK-C receptor is known to be the C-terminal tetrapeptide, so N-truncated peptides are expected to be suitable. For example, [Leu^{15}] gastrin$_{17}$ and [Nle^{15}] gastrin$_{2,17}$ have equal ability to bind to isolated canine parietal cells or parietal cell plasma membranes: substitution of methionine by leucine isomers does not interfere with the binding or biological activity of gastrin. A particularly preferred gastrin peptide is [Nle^{15}] gastrin$_{2,17}$, a derivative which lacks the amino-terminal pyroglutamate residue. Substitution of nor-leucine for the naturally occurring methionine at position 15 prevents formation of oxidised methionine derivatives during iodination. Although [Met^{15}]-gastrin$_{2,17}$ could be used, there would be some loss of activity for this reason. It is contemplated that gastrin peptides extended at the C-terminus could also be used.

In another preferred embodiment, a displacement-type assay using reversible binding is employed. The ability of the putative antagonist to prevent binding of a known agonist or antagonist of very high affinity is measured, said known agonist or antagonist being labelled with a detectable marker. Preferably the known agonist or antagonist has at least the binding affinity of benzotript. The known antagonist is suitably labelled with ^{3}H.

The method of the invention provides a general screening method for identifying antagonists of the gastrin-gastrin binding protein interaction, which compounds are useful for treatment of diseases, especially neoplastic diseases, involving rapidly proliferating cells. Such antagonists are also useful for controlling gastric acid secretion.

One preferred group of compounds useful for prevention and treatment of neoplastic diseases is non-

steroidal anti-inflammatory drugs (NSAIDS), which are especially useful for treatment of neoplastic diseases of the gastrointestinal tract, in particular colorectal cancer. However, we have shown that GBP is widespread in the body, and consequently treatment of cancers of non-gastrointestinal origin is also within the scope of this invention. It is preferable, but not essential, that the NSAID is an inhibitor of prostaglandin activity.

In another preferred embodiment, inhibition of the enoyl CoA hydratase and/or 3-hydroxyacyl CoA dehydrogenase activities intrinsic to the GBP is assayed. Either activity can be measured in either direction, but is conventionally assayed spectrophotometrically by the decrease in enoyl CoA absorption on hydration or by the decrease in NADH absorption on oxidation. Alternative assays could employ radiochemically labelled substrates.

Gastrin-binding protein suitable for use in the method of the invention may be partially purified or purified naturally occurring GBP, or may be recombinant GBP.

As an alternative to using a high-affinity labelled antagonist in a reversible binding assay, a high-affinity antibody, such as a monoclonal antibody, labelled with a detectable marker may be used. For example, it is contemplated that an antibody capable of blocking binding of an acyl CoA or of gastrin to GBP may be used.

The invention in another aspect therefore provides a method of treating neoplastic disease, comprising the step of administering to a mammal in need of such treatment an effective amount of a compound having the ability to inhibit binding of a gastrin peptide to gastrin-binding protein.

Preferably the neoplastic disease is selected from the group consisting of gastrointestinal cancers such as colon carcinoma and gastric carcinoma, mammary carcinoma, malignant melanoma, tumours of epithelial origin, and lymphomas.

In yet another aspect, the invention provides a pharmaceutical composition of a compound with the ability to inhibit the binding of a gastrin analogue to the low-affinity gastrin-cholecystokinin type C receptor, comprising a pharmaceutically-effective amount of said compound and a pharmaceutically-acceptable carrier.

In one preferred embodiment the compound is a non-selective antagonist of the gastrin-cholecystokinin receptor. Preferably the antagonist will have a IC_{50} value in the millimolar range or lower.

The compositions of the invention may comprise an antagonist either alone, or in combination with other pharmaceutically-active molecules such as antacid compounds or anti-cancer agents. Anti-cancer agents may include cytokines. Methods and pharmaceutical carriers for preparation of pharmaceutical compositions are well known in the art, as set out in text books such as Remington's Pharmaceutical Sciences, 17th Edition, Mack Publishing Company, Easton, Pennsylvania, USA.

In a fourth aspect, the invention provides a method of treatment of a neoplastic disease, comprising the step of inducing production of antisense gastrin mRNA in neoplastic tissue of an animal in need of such treatment. Tissue-specific production of anti-sense mRNA can be achieved by targeting of corresponding DNA to the desired tissue, using tissue-specific promoters.

DETAILED DESCRIPTION OF THE INVENTION

The invention is illustrated by reference to the following non-limiting examples, and to the figures, in which:

Figure 1 shows gastrin analogues and antagonists inhibit covalent cross-linking of ^{125}I-[Nle^{15}]-gastrin$_{2,17}$ to the 78kDa GBP.

Cross-linking of ^{125}I-[Nle^{15}]-gastrin$_{2,17}$ to the 78 kDa GBP in the presence of increasing concentrations of (A) gastrin analogues (closed symbols) and (B) gastrin/CCK

receptor antagonists (open symbols) was measured by phosphorimager (insets), and expressed as a percentage of the value obtained in the absence of competitor. The following values for IC_{50} and for the predicted ordinate intercept were obtained with the program LIGAND: $gastrin_{17}$ (♦), 0.11 μM, 102%; CCK_8 (■), 5.6 μM, 98%; CCK_8SO_4 (●), 3.55M, 125%; pentagastrin (▲), 200 μM, 96%; $gastrin_4$ (▼), 320 μM, 98%; proglumide (□), 4.1 mM, 94%; benzotript (O), 250 μM, 114%. Lines of best fit are not shown for CCK_8 or $gastrin_4$ for clarity. No inhibition was observed with the CCK-A receptor-selective antagonist L364,718 (data not shown), or with the gastrin/CCK-B receptor-selective antagonist L365,260 (◊).

Figure 2 shows binding of fatty acyl CoA derivatives to the 78 kDa GBP.

Cross-linking of ^{125}I-[Nle^{15}]-$gastrin_{2,17}$ to the 78 kDa GBP was measured by phosphorimager (insets) in the presence of increasing concentrations of crotonyl CoA, a substrate of enoyl CoA hydratase, or of acetoacetyl CoA, a product of 3-hydroxyacyl CoA dehydrogenase, and expressed as a percentage of the value obtained in the absence of competitor. Lines of best fit for inhibition by acetoacetyl CoA and crotonyl CoA were obtained with the program LIGAND. Values for IC_{50} and for the predicted ordinate intercept were as follows: Crotonyl CoA (●), 43 μM, 76%; acetoacetyl CoA (■), 74 μM, 70%. No inhibition was observed with NADH (▲), a cofactor for 3-hydroxyacyl CoA dehydrogenase.

Figure 3 shows that anti-proliferative gastrin/CCK receptor antagonists target the 78 kDa GBP. The mean IC_{50} values (Table 1) for the inhibition of cross-linking of ^{125}I-[Nle^{15}]-$gastrin_{2,17}$ to the 78 kDa GBP by gastrin analogues (closed symbols as in Figure 2) and antagonists (open symbols as in Figure 2) were determined as described in the legend to Figure 2, and compared with the IC_{50} values for (A) inhibition of binding of ^{125}I-CCK_8 labelled using Bolton-Hunter reagent to the cloned human

gastrin/CCK-B receptor expressed in COS cells (analogues) (8) and of ^{125}I-CCK$_{33}$ to the gastrin/CCK-B receptor on guinea pig gastric glands (antagonists) (9), or (B) inhibition of binding of ^{125}I-gastrin$_{17}$ to the gastrin/CCK-C receptor on HCT 116 colon carcinoma cells (2). Lines of best fit were obtained by linear regression.

Figure 4 illustrates processing and sequences of gastrin-related peptides.

The C-terminal sequence of human progastrin is shown using the one letter code. The corresponding region of porcine progastrin is only shown where it differs from human progastrin. The indicated peptides (numbered from the N-terminus of gastrin$_{1-17}$) were synthesised according to the human sequence, with the N-terminal glutamic acid of gastrin$_{1-4}$, gastrin$_{1-11}$, gastrin$_{1-17}$ and gastrin$_{1-17}$G cyclized to pyroglutamate. The C-termini of gastrin$_{1-17}$ and gastrin$_{14-17}$ were amidated.

Figure 5 shows binding of gastrin-related peptides to the 78 kDa GBP.

Cross-linking of gastrin-related peptides was measured as described above, and the cross-linking quantitated by phosphorimager, as shown in the inset panels, and lines of best fit calculated and plotted against peptide concentration.

Figure 6 illustrates how expression of antisense gastrin inhibits cell proliferation.

Proliferation of YAMC (A) or LIM 1215 (B) cells transfected with plasmid alone in the absence (black bars) or presence (light grey bars) of 1 μM gastrin$_{17}$, or with a plasmid expressing antisense gastrin mRNA in the absence (dark grey bars) or presence (white bars) of 1 μM gastrin$_{17}$ was measured with the MTT assay at the indicated times after seeding in media containing 1% fetal calf serum. The means of triplicate absorbance readings were expressed as a percentage of the mean absorbance reading obtained on day 1 for the YAMC cells grown in the absence of gastrin$_{17}$; error bars represent the standard error of the mean. Statistical

significance was assessed by Student's t-test: $p < 0.05$, *; $p < 0.02$, **.

Figure 7 shows the inhibition of YAMC cell proliferation by gastrin/CCK receptor antagonists.

Proliferation of YAMC cells in the presence of increasing concentrations of the non-selective gastrin/CCK receptor antagonists proglumide (●) or benzotript (■) was measured with the MTT assay at two days after seeding. Antagonists were added one day after seeding. The means of triplicate absorbance readings are shown as a percentage of the mean absorbance reading obtained on day 1 for the cells grown in 1% fetal calf serum; error bars represent the standard error of the mean. Lines of best fit were obtained with the programs EBDA and LIGAND as described previously (6).

Figure 8 shows the inhibition of growth of LIM 1215 and LIM 2412 cells by proglumide.

Uptake of ^{3}H-thymidine (O) by, and viability (□) of, cells was measured after 17 hours treatment with the concentration of proglumide indicated. LIM 1215 (A) and LIM 2412 (B) cells were grown in the absence of serum. Points are the mean values of 5 samples, and lines of best fit were obtained with the program LIGAND. Bars represent 1 SD.

Figure 9 illustrates proliferation of LIM 1215 colon carcinoma cells in the presence of increasing concentrations of NSAIDS, measured with the MTT assay as described for Figure 7. Cells were grown in a medium containing 10% foetal calf serum, and NSAIDS were added one day after seeding. The means of triplicate absorbance readings were expressed as a percentage of the mean absorbance reading obtained for cells grown in the absence of NSAIDS; error bars represent the standard error of the mean. Lines of best fit were obtained with the programs EBDA and LIGAND as described previously (6). Values for IC_{50} and for the predicted ordinate intercept were as follows: indomethacin, 91 μM, 137% (●); sulindac

sulphoxide, 358 μM, 132% (■); aspirin, 3.22 mM, 134% (▲). Similar results were obtained in 3 separate experiments.

Figure 10 shows that the inhibition of proliferation of LIM 1215 cells by NSAIDS is not correlated with the ability of the NSAIDS to inhibit either cyclooxygenase I (Panel A) or cyclooxygenase II (Panel B). The IC_{50} values for inhibition of proliferation of LIM 1215 cells were measured as described for Figure 8, and are summarized in Table 4. IC_{50} values for inhibition of cyclooxygenase I and cyclooxygenase II were taken from Mitchell *et al* (Proc. Natl. Acad. Sci. USA., 1994 90 11693-11697).

Figure 11 shows the ability of four different NSAIDS to block cross-linking of $gastrin_{2,17}$ to GBP in the assay described above.

Figure 12 shows the correlation between the ability of NSAIDS to inhibit proliferation of LIM 1215 cells and their ability to inhibit cross-linking of $gastrin_{2,17}$ to GBP.

Abbreviations used herein are as follows:

ASP	-	aspirin
CCK	-	cholecystokinin
CHO	-	Chinese hamster ovary
DIC	-	diclofenac
DIF	-	diflunisal
FCS	-	foetal calf serum
GBP	-	gastrin-binding protein
IBU	-	ibuprofen
IC_{50}	-	concentration required for 50% inhibition of cross-linking
IFN	-	niflumic acid
IND	-	indomethacin
MEC	-	meclofenamic acid
MEF	-	mefenamic acid
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2-,5-diphenyltetrazolium bromide
NAP	-	naproxen

PHE - phenylbutazone
PIR - piroxicam
SAL - salicylic acid
SUL - sulindac sulphoxide
SUS - sulindac sulphide
TIA - tiaprofenic acid
TOL - tolmetin
YAMC - young adult mouse colon.

MATERIALS AND METHODS

Gastrin$_{17}$, CCK$_8$, and CCK$_8$SO$_4$ were from Research Plus, Bayonne, NJ. Pentagastrin, gastrin$_4$, proglumide (4-(benzoylamino)-5-(dipropylamino)-5-oxopentanoic acid, acetoacetyl CoA, crotonyl CoA, pepstatin, benzamidine, hexamethylphosphoramide, aprotinin and NSAIDS were from Sigma, St.Louis, MO. Benzotript (N-(p-chlorobenzoyl)-L-tryptophan) was from Calbiochem, San Diego, CA. The antagonists L364,718 and L365,260 were generous gifts from Dr. V.J.Lotti, Merck, Sharp and Dohme, West Point, PA. Na^{125}I was from NEN, North Ryde, Australia.

^{125}1-[Nle^{15}]-gastrin$_{2,17}$ was prepared and iodinated as described in reference 5, the whole contents of which publication are herein incorporated by reference.

The 78 kDa GBP was partially purified from detergent extracts of porcine gastric mucosal membranes (5), and crosslinked to ^{125}I-[Nle]^{15}-gastrin$_{2,17}$ with disuccinimidylsuberate as previously described (5). The following protease inhibitors were included in all buffers to prevent proteolysis: pepstatin, 1 μM; benzamidine, 1 mM; hexamethylphosphoramide, 0.1% (w/v); aprotinin, 500 units/ml. The products of the cross-linking reaction were separated by polyacrylamide gel electrophoresis, and radioactivity associated with the 78 kDa GBP was detected and quantitated with a phosphorimager (Molecular Dynamics, Sunnyvale, CA). Initial estimates of IC$_{50}$ values, and of the levels of ^{125}I-gastrin$_{2,17}$ in the absence of competitor, were obtained by fitting the data with the program EBDA,

and were refined with the program LIGAND (3).

The purity and composition of gastrin-related peptides was assessed by the manufacturer (Chiron Mimotopes, Clayton, Australia) by reversed phase high pressure liquid chromatography and by ion spray mass spectrometry.

Human gastrin cDNA in the plasmid pBR322 (18) was kindly provided by Dr. E. Boel, University of Aarhus, Denmark. The conditionally immortalised young adult mouse colon cell line (YAMC), which was derived from a mouse transgenic for a temperature sensitive SV40 large T antigen (19), and the human colon carcinoma cell line LIM 1215, which was derived from a patient with inherited nonpolyposis colorectal cancer (20), were obtained from Dr. R.H. Whitehead, Ludwig Institute for Cancer Research, Melbourne.

Cell Proliferation Assay.

A colorimetric assay (21) was used to measure cell proliferation. Briefly, 10^4 cells were seeded in a 96 well plate in medium containing 1% fetal calf serum. The medium was replaced the next day with fresh medium containing 1% fetal calf serum and the substance under investigation. For each time point 10 µl of 5 mg/ml MTT

(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, St.Louis, MO) was added to each well, and the plate was incubated for 4 hrs at 37°C before the medium was discarded. 200 µl 0.04M HCl in isopropanol was added to lyse the cells, and the absorbance at 560 nm was read on a Titertek Multiscan MCC 1340 (Labsystems, Helsinki, Finland). Student's t-test was used for statistical analysis.

In one set of experiments with the human colon carcinoma cell lines LIM 1215 and LIM 2412, cell proliferation was measured using a ^{3}H-thymidine uptake assay, using a standard method. LIM 2412 cells were grown in RPMI 1640 medium, and LIM 1215 cells in the same medium containing 1 mM thioglycerol, 25 units/ml insulin, 1 mg/ml hydrocortisone, 5 mg/ml bovine serum albumin, 10 µg/ml iron-saturated transferrin and 1.5 mg/ml glutamine. Both cell lines were cultured in the absence of serum. Proglumide (200 mM, Sigma, St. Louis, MO) was made up in 50 mM Na^+ HEPES, pH 7.6, readjusted to pH 7.6 with 10 M NaOH, and added at concentrations ranging from 1 to 50 mM. Control wells received the same volume of 50 mM Na^+ HEPES, pH 7.6, as this buffer was sometimes found to inhibit [^{3}H]-thymidine uptake. The cells were then incubated in a total volume of 100 µl at 37°C in a humidified atmosphere of 10% CO_2. After 17 hours the cells were pulsed with [^{3}H]-thymidine (0.5 µCi per ml, NEN-DuPont, Boston, MA) for four hours. The medium was removed, and the cells were lysed with a mixture of 30 µl lysis buffer (30 mM Tris HCl, pH 7.6, 100 mM EDTA, 1% w/v Sarcosyl (Sigma, St. Louis, MO)) and 70 µl PBS, harvested and counted using a Beta-Plate harvester and liquid scintillation counter (LKB-Wallace, Turku, Finland). Cell viability in a duplicate plate was measured by Trypan Blue exclusion.

Radioimmunoassay

The gastrin concentrations in conditioned media and cell extracts were determined by radioimmunoassay

against an amidated gastrin$_{17}$ standard curve with ^{125}I-amidated gastrin$_{17}$ as label (22). The calculated 50% inhibitory dose for antiserum 8017 was 1.6 $\pm$ 0.1 fmol/tube (n=13). The intraassay variation was <6% (n=6) and the interassay variation was less than 20% (n=13). Levels of amidated gastrin were assayed with antiserum 1296, which detects all amidated carboxyl terminal fragments greater than the pentapeptide, and does not cross-react with glycine-extended forms. Levels of unprocessed, partially processed and mature amidated forms of gastrin (i.e. all progastrin-derived peptides) were measured with antiserum 8017, which detects the amino terminal portion of gastrin$_{17}$, after digestion of samples with trypsin (23). Samples were treated with an equal volume of 50 µg/ml trypsin in 20 mM veronal, pH 8.2, containing 0.005% sodium azide and 2% charcoal-stripped plasma. After incubation at 37°C for 2 hr, the samples were boiled for 2 min, cooled on ice for 2 min, and frozen until assay.

Gastrin Binding Assays

Gastrin/CCK-B receptors were measured by the method of Kopin *et al* (24). 10^4 cells/well were seeded in a 24 well plate and grown for 2 days at 33°C in RPMI medium containing 10 mM thioglycerol, 100 units/ml insulin, 50 mg/ml hydrocortisone and 10% fetal calf serum. Cells were washed in phosphate-buffered saline, and incubated for 80 min at 37°C in Hank's balanced salt solution containing ^{125}I-CCK$_8$ (10,000 cpm, 2.9 fmol, Amersham, Bucks., UK), 16 µM PMSF and 0.1% BSA. Cells were then washed twice with PBS and lysed with NaOH. Lysates were counted in a γ-counter (Packard, Downer's Grove, IL) at 77% efficiency.

Gastrin/CCK-C receptors were measured by the phthalate oil centrifugation method (3) with ^{125}I-gastrin$_{17}$ (1.5 x 10^5 cpm, 44 fmol (Amersham, Bucks., UK)) as ligand.

Example 1 Definition of the Gastrin Binding Site

The ability of gastrin peptides and of non-specific and specific CCK receptor antagonists to inhibit cross-linking of GBP to a labelled gastrin peptide was tested as described above. The results, expressed as IC_{50} values, are set out in the far right hand column of Table 1, and are compared with IC_{50} values previously reported in the literature for binding of gastrin agonists or antagonists to tissues or tumour cells, or for inhibition of proliferation of tumour cell lines. Values are for the human receptors unless indicated as follows: [a] guinea pig; [b] rat. ND, not determined. Reference numbers are given in brackets.

IC_{50} values (mean ± SD, calculated from at least 3 sets of data) for inhibition of cross-linking of ^{125}I-[Nle^{15}]-gastrin$_{2,17}$ to the 78 kDa GBP in detergent extracts of porcine gastric mucosal membranes were determined from the data presented in Figure 1 and from similar experiments by least squares fitting with the programs EBDA and LIGAND.

Gastrin$_{17}$ and its derivatives pentagastrin and gastrin$_4$ all inhibited cross-linking of ^{125}I-[Nle^{15}]-gastrin$_{2,17}$ to the 78 kDa GBP, as illustrated in Figure 1A. The observed IC_{50} values, which are dependent on chain length, are in good agreement with the IC_{50} values reported previously for inhibition of binding of ^{125}I-gastrin$_{17}$ to the gastrin/CCK-C receptor on the HCT 116 colon carcinoma cell line (see Table 1). In contrast, the IC_{50} values for the 78 kDa GBP differ markedly from the IC_{50} values for the CCK-A and gastrin/CCK-B receptors shown in Table 1. The correlation between the IC_{50} values for the 78 kDa GBP and the IC_{50} values for the gastrin/CCK-C receptor, and the lack of correlation between the IC_{50} values for the 78 kDa GBP and the IC_{50} values for the gastrin/CCK-B receptor, is particularly apparent when the data are presented graphically (Figures 3A and 3B).

Example 2 Inhibition by Gastrin/CCK Receptor Antagonists.

The non-selective antagonists proglumide and benzotript both inhibited cross-linking of ^{125}I-gastrin$_{2,17}$ to the GBP, as shown in Figure 1B, with IC_{50} values of 5.1 mM and 195 μM respectively (Table 1). As with gastrin and its derivatives, a good correlation is observed between the IC_{50} values for the 78 kDa GBP and the IC_{50} values for inhibition of ^{125}I-gastrin$_{17}$ binding to the gastrin/CCK-C receptor, while the correlation between the IC_{50} values for the 78 kDa GBP and the IC_{50} values for the gastrin/CCK-B receptor is poor. These results are summarised in Figure 3. Furthermore, neither the CCK-A receptor antagonist L364,718 nor the gastrin/CCK-B receptor antagonist L365,260 had any effect on cross-linking at concentrations as high as 200 μM (Figure 1B). A good correlation was also observed between the IC_{50} values for the 78 kDa GBP and the IC_{50} values for inhibition of HCT 116 and HT 29 colon carcinoma cell proliferation by proglumide and benzotript (Table 1).

Example 3 Inhibition by Fatty Acid Derivatives.

A substrate for enoyl CoA hydratase, crotonyl CoA, and a product of 3-hydroxyacyl CoA dehydrogenase, acetoacetyl CoA, both inhibited cross-linking of ^{125}I-gastrin$_{2,17}$ to the GBP, as summarised in Figure 2. The mean IC_{50} values from 3 separate experiments were 45 ± 26 µM for crotonyl CoA and 180 ± 150 µM for acetoacetyl CoA. No inhibition was observed with NADH, the cofactor of the dehydrogenase reaction, at concentrations as high as 1.5 mM.

Example 4 Amidation of Gastrin$_{17}$ is not Required for GBP Binding

The sequences of gastrin related peptide and glycine-extended peptides are shown in Figure 4.

Processing of progastrin to form gastrin begins by cleavage by a dibasic-specific endopeptidase at the sites marked by arrowheads. Removal of the basic amino acids by carboxypeptidase E yields glycine-extended gastrin$_{17}$ (gastrin$_{1-17}$G), which is transamidated by peptidyl-glycine α-amidating monooxygenase to form mature amidated gastrin$_{1-17}$.

The effect of extending the C-terminus of gastrin$_{17}$ on binding to the GBP was investigated by comparing the potencies of glycine-extended gastrin$_{17}$ and gastrin$_{17}$ as inhibitors of cross-linking of [^{125}I]-Nle^{15}-gastrin$_{2-17}$ to the GBP.

IC_{50} values (mean ± SEM of at least 3 separate determinations) for the inhibition of cross-linking of [^{125}I]-Nle^{15}-gastrin$_{2-17}$ to the 78 kDa gastrin-binding protein by the indicated peptides were determined as described above, and are summarised in Table 2. IC_{50} values for gastrin$_{12-17}$GRRSAE, gastrin$_{1-4}$ and YFGRRSAEEGD represent estimates only, because the limiting amounts of peptide available precluded assay at concentrations greater than 100, 400 and 100 µM respectively. IC_{50} values for the gastrin/CCK-B receptor on canine parietal cells, and for

the binding of gastrin$_{1-17}$ and gastrin$_{14-17}$ to the GBP, are taken from reference 24 and Example 1 respectively. The amino acid sequences of C-terminal extensions are shown in the one letter code; the peptide sequences are shown in full in Figure 4.

Table 2

Affinities of Gastrin-Related Peptides for Gastrin Receptors

Peptide	Purity (%)	IC_{50} (μM) 78 kDa Gastrin-Binding Protein	IC_{50} (μM) Gastrin/CCK-B * Receptor
Gastrin$_{1-17}$	82	0.23 ± 0.15	5 x 10^{-4}
Gastrin$_{1-17}$G	78	0.19 ± 0.10	ND
Gastrin$_{5-17}$G	93	26 ± 15	ND
Gastrin$_{5-17}$GRRSAE	87	20 ± 11	ND
Gastrin$_{12-17}$G	81	31 ± 17	14 ± 71
Gastrin$_{12-17}$GRR	ND	ND	44 ± 21
Gastrin$_{12-17}$GRRSAE	91	760 ± 550	ND
Gastrin$_{14-17}$	95	370 ± 280	0.1
Gastrin$_{1-11}$	97	21 ± 9	ND
Gastrin$_{1-4}$	98	>1000	ND
YFGRRSAEEGD	62	105 ± 21	ND

ND : not determined * Values taken from Reference 11

The IC_{50} value for glycine-extended gastrin$_{17}$ was slightly lower than the IC_{50} value for gastrin$_{17}$, as shown in Figure 5 and Table 2. Addition of a further 5 residues to the C-terminus of glycine-extended gastrin$_{5-17}$ did not alter the affinity of the resultant peptide for the GBP, while addition of 5 residues to the C-terminus of glycine-extended gastrin$_{12-17}$ actually resulted in a 25-fold reduction in affinity.

Table 1

Comparison of the 78 kDa GBP with Human Gastrin and Cholecystokinin Receptors.

	IC_{50} for Binding (μM)			IC_{50} for Cell Proliferation (μM)		IC_{50} for Cross linking (μM)
Receptor Tissue	CCK-A Pancreas, Gall Bladder	Gastrin/CCK-B Stomach, Brain	Gastrin/CCK-C HCT 116 Cells	HCT 116 Cells	HT 29 Cells	78 kDa GBP Stomach
Agonists						
$Gastrin_{17}$	0.08 1.8	6.4×10^{-3} 9.4×10^{-4} 2.6×10^{-3}	1.6	-	-	0.23 ± 0.15
CCK_8	0.14[a]	1.9×10^{-3} 4.7×10^{-3}	13.2	-	-	4.5 ± 1.0
CCK_8SO_4	2×10^{-5} 3×10^{-3}	3×10^{-3} 1.4×10^{-4} 1.1×10^{-4}	ND	-	-	5.8 ± 3.2
Pentagastrin	ND	ND	100	-	-	210 ± 90
$Gastrin_4$	ND	3.2×10^{-2} 3.0×10^{-2}	ND	-	-	370 ± 280
Antagonists						
Proglumide	600[b]	900[a]	8600	5000	14000	5100 ± 3600
Benzotript	102[b]	59[a]	400	500	3400	200 ± 120
L364,718	1×10^{-4} 8×10^{-4}	0.5 0.15	ND	4	>100	>200
L365,260	9 0.02	1.0×10^{-2} 3.8×10^{-3}	ND	15	22	>200

Data in first 5 columns summarised from reports in the literature.

Example 5 Gastrin$_{17}$ Binding Determinants

The regions of gastrin$_{17}$ contributing to GBP binding were investigated by testing the effect of N- and C-terminal deletion on the potency of glycine-extended gastrin$_{17}$ as an inhibitor of cross-linking of [^{125}I]-Nle^{15}-gastrin$_{2-17}$ to the GBP. Results are shown in Figure 5.

Cross-linking of ^{125}I-[Nle^{15}]-gastrin$_{2-17}$ to the 78 kDa GBP was measured as described above in the presence of increasing concentrations of gastrin-related peptides. Duplicate samples were subjected to electrophoresis on NaDodSO$_4$-polyacrylamide gels, and the radioactivity associated with the 78 kDa GBP was quantitated by phosphorimager scanning and expressed as a percentage of the value obtained in the absence of competitor. The following values for IC_{50} and for the predicted ordinate intercept were obtained by computer fitting as described herein, and used to construct the indicated lines of best fit: (A) gastrin$_{1-17}$ (138 nM, 115.5%) or glycine-extended gastrin$_{1-17}$ (98 nM, 90.0%), (B) glycine-extended gastrin$_{5-17}$ (11 μM, 93.8%) or glycine-extended gastrin$_{12-17}$ (40 μM, 99.6%), or (C) gastrin$_{1-4}$ (1.96 mM, 101.7%) or gastrin$_{1-11}$ (31 μM, 101.3%).

Comparison of the affinities of gastrin analogues for the 78 kDa GBP revealed that both ends of gastrin$_{17}$ contribute to binding. Thus removal of 4 residues (pyroglutamylGPW) from the N-terminus of glycine-extended gastrin$_{17}$ resulted in a 140-fold decrease in affinity, while removal of 7 residues (YGWMDFG) from the C-terminus of glycine-extended gastrin$_{17}$ resulted in a 110-fold decrease in affinity (Table 2). Removal of a further 7 residues from the C-terminus of glycine-extended gastrin$_{17}$ resulted in almost complete loss of binding affinity. In reciprocal experiments the binding of the deleted peptides to the GBP was also measured. As expected, gastrin$_{14-17}$ bound weakly to the GBP, but binding of the N-terminal tetrapeptide pyroglutamylGPW was barely detectable. Since benzotript, which we have shown in Example 1 to be the best

available antagonist for the GBP, is an acylated tryptophan derivative (N-4-chlorobenzoyl-L-tryptophan), it seems likely that the tryptophan residue of either the N-terminal or C-terminal tetrapeptide, or both, makes a significant contribution to binding.

The role of the sequence of five glutamic acid residues of $gastrin_{17}$ in GBP binding is not clear at present. The similar affinities of glycine-extended $gastrin_{5-17}$ and glycine-extended $gastrin_{12-17}$ for the GBP (Table 2) suggest that this polyglutamate sequence makes no contribution. However, deletion of the five glutamate residues from $gastrin_{1-11}$ to yield $gastrin_{1-4}$ resulted in a 60-fold decrease in affinity.

In contrast to the present results with the GBP, two lines of evidence demonstrate that the gastrin/CCK-B receptor does not recognise the N-terminus of $gastrin_{17}$. Firstly, no binding of the N-terminal tridecapeptide to the gastrin/CCK-B receptor on canine parietal cells was observed even at concentrations as high as 100 μM (11). Secondly, removal of 4 residues from the N-terminus of $gastrin_{17}$ had no effect on binding to rabbit parietal cells (12). The observation that removal of a further 5 residues, including 4 of the 5 glutamic acid residues, reduced binding to rabbit parietal cells by 100-fold additionally implied that the pentaglutamic acid sequence contributed to gastrin/CCK-B receptor binding (16). The gastrin binding sites of the GBP and the gastrin/CCK-B receptor are thus clearly distinct.

Example 6 Anti-Sense Progastrin RNA Inhibits Expression of Progastrin and Gastrin

A cDNA fragment encoding human progastrin (18) was cut from pBR322 with PstI and cloned into pGEM-3Z (Promega, Madison, WI). An expression plasmid encoding gastrin mRNA in the sense orientation was then constructed by cloning a Bam HI/SphI fragment from pGEM-3Z into pCDNA1amp (Invitrogen, San Diego, CA). The corresponding

antisense plasmid was constructed by cloning an XbaI/SphI fragment from pGEM-3Z into pCDNA1amp, and a XbaI/BstXI fragment thence into pCDNA1neo (Invitrogen).

For transient transfections 10^7 COS cells were incubated with 2.5 µg/ml plasmid DNA, 100 µM chloroquine and 200 µg/ml DEAE-dextran in RPMI medium containing 2 mM glutamine and 10% heat-inactivated NuSerum (Gibco/BRL, Gaithersburg, MD) for 4 hr. at 37°C, grown for 3 days at 37°C in RPMI medium containing 2 mM glutamine and 10% fetal calf serum, and harvested. The lipofection method (25) was used to generate stable cell lines. Half-confluent YAMC cells were incubated with 20 µg/ml plasmid DNA for 4-6 hrs at 33°C in serum-free RPMI medium containing 20 µg/ml lipofectin (Gibco/BRL), 10 mM thioglycerol, 100 units/ml insulin and 50 mg/ml hydrocortisone. Transfected cells were grown at 33°C in RPMI medium containing 10 mM thioglycerol, 100 units/ml insulin, 50 mg/ml hydrocortisone and 10% fetal calf serum for 2 days, and selected for 14 days in the same medium containing 100 µg/ml neomycin.

No progastrin or amidated gastrin was detected in YAMC or LIM 1215 cell extracts and conditioned media. In contrast, progastrin-derived peptides were readily detected in YAMC cell extract (20 fmol/10^7 cells) and conditioned medium (66 fmol/10^6 cells) following transfection with a sense gastrin construct; however, amidated gastrin was not detected in these cells. No progastrin-derived peptides were detected in YAMC or LIM 1215 cells transfected with an antisense gastrin construct.

To confirm that expression of antisense gastrin mRNA was able to reduce levels of progastrin-derived peptides, COS cells were transiently transfected with a plasmid encoding sense gastrin mRNA or with plasmids encoding both sense and antisense gastrin mRNA. The levels of progastrin-derived peptides in cell extracts (760 pmol/10^7 cells) and conditioned media (1540 pmol/10^7 cells) were reduced by 37 and 48% respectively in the doubly transfected cells. Amidated gastrin was not detected in

either case. Similar results were obtained in 3 separate experiments with YAMC, and two experiments with LIM 1215.

We have previously demonstrated the presence of very low levels (2.1 molecules/1000 cells) of gastrin mRNA in LIM 1215 cells by quantitative PCR (15). Unfortunately we have been prevented from quantitating the amount of gastrin mRNA in YAMC cells by PCR by absence of a published mouse gastrin cDNA sequence. However, we have been unable to detect any progastrin-derived peptides or mature amidated gastrin by radioimmunoassay in either cell extracts or media conditioned by YAMC or LIM 1215 cells. Based on the sensitivity of the assay (0.4 fmol), we should have detected levels of progastrin-derived peptides as low as 1 fmol/10^6 cells. Presumably the discrepancy between our results and the observation that progastrin-derived peptides were present in all 5 colon carcinoma cell lines tested at levels between 17 and 54 fmol/10^6 cells (13) is a reflection of the fact that LIM 1215 cells had the lowest levels of gastrin mRNA of 7 colon carcinoma cell lines tested (15). Because of the absence of detectable levels of progastrin-derived peptides in YAMC and LIM 1215 cells, we were unable to demonstrate any reduction in levels following expression of antisense gastrin mRNA. However, we were able to demonstrate such a reduction in COS cells transiently transfected with plasmids expressing both sense and antisense gastrin mRNA, compared with COS cells transiently transfected with plasmids expressing sense gastrin mRNA only. We therefore postulate that the observed inhibition of proliferation of YAMC and LIM 1215 cells is a consequence of a reduction in the level of progastrin-derived peptides.

Example 7 Expression of Antisense Gastrin Reduces Proliferation

Expression of antisense gastrin mRNA significantly reduced proliferation of both YAMC cells and the colon carcinoma cell line LIM 1215 (Figure 6). In

neither case was the inhibition reversed by the addition of $gastrin_{17}$ to the medium.

Example 8 Gastrin Receptors in YAMC and LIM 1215 cells

The total amount of ^{125}I-CCK_8 (10,000 cpm, 2.9 fmol) bound to gastrin/CCK-A + -B receptors on YAMC, LIM 1215 and AR4-2J cells was measured by the method of Kopin *et al* (24). The AR4-2J cell line was derived from a rat pancreatic carcinoma, and expresses both gastrin/CCK-A + -B receptors. The amount of ^{125}I-$gastrin_{17}$ (150,000 cpm, 44 fmol) bound to gastrin/CCK-C receptors on YAMC and LIM 1215 cells was measured by the phthalate oil centrifugation assay described by Weinstock and Baldwin (3). Background binding was measured in the presence of 1 μM (A + B) or 10 μM (C) $gastrin_{17}$. The results are summarised in Table 3.

Table 3

Presence of Gastrin/CCK-C, but not Gastrin/CCK-A or -B Receptors on YAMC and LIM 1215 Cells

Cell line	Cell No.	^{125}I-CCK_8 Bound (cpm)	Background (cpm)
Gastrin/CCK-A + -B Receptors			
YAMC	10^4	201 ± 22	171 ± 12
LIM 1215	10^4	1663 ± 89	1604 ± 106
AR4-2J	10^4	10567 ± 1341	1866 ± 252
Gastrin/CCK-C Receptors			
YAMC	4.2×10^5	1002 ± 72	420 ± 88
LIM 1215	10^6	5407 ± 800	700 ± 89

No CCK-A or gastrin/CCK-B receptors were detected on YAMC or LIM 1215 cells by binding of ^{125}I-CCK_8, even though gastrin/CCK-A and -B receptors were readily detected on AR4-2J cells. The lack of involvement of either CCK-A or gastrin/CCK-B receptors in the autocrine growth of YAMC cells was confirmed by demonstrating that neither the CCK-

A-receptor selective antagonist L364,718, nor the gastrin/CCK-B receptor-selective antagonist L365,260, inhibited YAMC cell proliferation at concentrations (100 nM) sufficient to saturate their respective receptors (data not shown).

Gastrin/CCK-C receptors were readily detected on both YAMC and LIM 1215 cells using the phthalate oil centrifugation assay and ^{125}I-gastrin$_{17}$ as ligand.

Example 9 Inhibition of Cell Proliferation by Receptor Antagonists

The effect of non-selective gastrin/CCK receptor antagonists on the proliferation of YAMC cells was measured. The non-selective gastrin/CCK receptor antagonists proglumide and benzotript were both able to inhibit proliferation of YAMC cells, as assessed using the MTT assay at two days after seeding of the cells. The results of one experiment are shown in Figure 7.

Values for IC_{50} and for the predicted ordinate intercept were as follows: proglumide, 7.4 mM, 119% ; benzotript, 13.7 mM, 113%. Concentrations of benzotript higher than 3 mM were toxic to YAMC cells. No inhibition of proliferation was observed with the CCK-A receptor-selective antagonist L364,718 or the gastrin/CCK-B receptor-selective antagonist L365,260 at 100 nM. Similar results were obtained in 2 further experiments.

Our overall results of inhibition of proliferation of YAMC by proglumide (IC_{50} value of 7.2 $\pm$ 0.4 mM) and by benzotript (IC_{50} value of 8.0 $\pm$ 8.1 mM) were consistent with the involvement of the gastrin/CCK-C receptor in the autocrine growth of YAMC. Similarly, we have found that proliferation of LIM 1215 cells and LIM 2412 cells is inhibited by proglumide, with IC_{50} values of 2.2 $\pm$ 1.0 mM, and 2.9 $\pm$ 1.3 mM respectively. No correlation was observed between the degree of inhibition by proglumide and the presence or absence of binding sites for ^{125}I-[Nle^{15}]-gastrin$_{17}$ determined previously. Even

though $gastrin_{17}$ breakdown did not exceed 30% during a 24 hour incubation in the absence of serum and a non-saturating concentration of inhibitor was chosen to facilitate ready reversal of inhibition, no reversal of the inhibition of LIM 1215 or LIM 2412 cells by proglumide was detected with $gastrin_{17}$ concentrations as high as 50 μM. These results are shown in Figure 8.

Example 10 Inhibition of Proliferation of LIM 1215 Cells by Non-Steroidal Anti-Inflammatory Drugs

NSAIDS have been shown to inhibit colorectal tumour growth in several experimental models, and have been shown to reduce the size and number of colorectal polyps in patients with familial adenomatous polyposis (26), and to reduce the death rate from cancer of the eosophagus, stomach, colon, and rectum (27). It has been widely accepted that the effect of NSAIDS on colon cancers has resulted from the ability of these agents to inhibit cyclooxygenases, which are key enzymes in the synthesis of prostaglandins, and in particular it has been proposed that the anti-tumour activity is correlated with inhibition of cyclooxygenase-2 (28).

We have compared the ability of a variety of NSAIDS to inhibit the proliferation of the colon carcinoma cell line LIM 1215 with their ability to inhibit cyclooxygenase I and cyclooxygenase II. Cell proliferation was measured using the colorimetric assay described above. For these experiments, the medium was RPMI 1640 containing 10 μM thioglycerol, 25 units/ml insulin, 1 mg/ml hydrocortisone and 10% foetal calf serum. After 24-hours, fresh medium containing the test drug was added. Otherwise the assay was as described above. Figure 9 shows results for indomethacin, sulindac sulphoxide and aspirin. All of these agents inhibited proliferation of LIM 1215 cells. In fact, all of the NSAIDS tested inhibited proliferation of LIM 1215 cells, with IC_{50} values obtained by computer curve

fitting with the programmes EBDA and LIGAND ranging from 120 μM for meclofenamic acid and sulindac sulphide to 21 mM for acetaminophen. These results are summarized in Table 4, and are also compared for some agents with IC_{50} values for cyclooxygenase I and cyclooxygenase II, which have been reported in the literature.

Table 4

IC_{50} Values for Inhibition of Colon Carcinoma Cell Proliferation by NSAIDS

NSAIDS	IC_{50} (μM)			
	LIM 1215 Cell Proliferation	GBP Cross-linking	Cyclo-oxygenase I	Cyclo-oxygenase II
Acetaminophen	21000 ± 14000	>10000	17.9*	132*
Aspirin	1700 ± 640	9300 ± 4700	1.7	278
Diclofenac	120 ± 110	160 ± 90	1.6	1.1
Ibuprofen	290 ± 160	2300 ± 600	4.8	73
Indomethacin	200 ± 130	150 ± 40	0.03	1.7
Naproxen	660 ± 460	1200 ± 400	9.6	5.7
Salicylate	1750 ± 400	3300 ± 1400	219	625
Diflunisal	160 ± 80	80 ± 30		
Meclofenamate	120 ± 70	90 ± 50		
Mefenamate	200 ± 60	220 ± 60		
Niflumate	130 ± 100	290 ± 180		
Phenylbutazone	600 ± 180	2200 ± 400		
Piroxicam	690 ± 390	2600 ± 500		
Sulindac S	120 ± 120	40 ± 10		
Sulindac	330 ± 170	580 ± 260		
Tiaprofenate	440 ± 230	1400 ± 400		
Tolmetin	1900 ± 170	1400 ± 100		

* IC_{30} values are shown because 50% inhibition of cyclooxygenase II was not achieved by concentrations as high as 1 mg/ml (28).

Figure 10 compares IC_{50} values for LIM 1215 cell proliferation and for inhibition of cyclooxygenases I and II in intact bovine aortic endothelial cells and murine macrophages respectively, and shows that there is no correlation between inhibition of cell proliferation and enzyme inhibition for either enzyme. In particular, the potent cyclooxygenase inhibitor indomethacin, which has an IC_{50} value of 30 nM for cyclooxygenase I and 1.7 μM for cyclooxygenase II, was only able to inhibit LIM 1215 cell proliferation to a moderate extent, having an IC_{50} value of 200 μM. Moreover, acetaminophen , which does not decrease the risk of gastrointestinal tract cancer (27), was even less effective in inhibiting LIM 1215 cell proliferation, having an IC_{50} of 21 mM. This agent is more potent than aspirin as an inhibitor of cyclooxygenase II, with an IC_{50} value of 130 μM (28). Our results provide further evidence that the anti-proliferative affects of NSAIDS on colon cancers are not primarily mediated by cyclooxygenase II.

Example 11 Non-Steroidal Anti-Inflammatory Drugs Inhibit Cross-Linking of Gastrin Analogue to Gastrin-Binding Protein

Using the cross-linking assay described above, the ability of several NSAIDS to inhibit the cross-linking of ^{125}I-gastrin$_{2,17}$ to gastrin-binding protein was examined. The results for sulindac sulphide, indomethacin, ibuprofen and aspirin are presented in Figure 11. All four agents inhibited the cross-linking in a concentration-dependent manner, with the order of activity being sulindac sulphide > indomethacin > ibuprofen > aspirin.

The correlation between ability to inhibit in the gastrin-gastrin binding protein cross-linking assay and the ability to inhibit proliferation of LIM 1215 cells was therefore investigated, using sixteen different NSAIDS. The results are shown in Figure 12. Anti-proliferative activity was strongly correlated with the IC_{50} value in the gastrin binding protein cross-linking assay, with a

correlation coefficient of 0.87.

Again the results suggested that the strongest activity was shown by sulindac sulphide, and the weakest by aspirin. No inhibition was detected with acetaminophen at concentrations as high as 10 mM.

We conclude that the gastrin-binding protein cross-linking assay is useful, either alone or in conjunction with the cell proliferation assay, to identify NSAIDS useful for treatment of diseases involving rapidly proliferating cells, especially neoplastic diseases. It is considered that use of both assays for a given candidate comopund gives a more accurate prediction of activity than the use of either assay alone.

CONCLUSIONS

Identity of the 78 kDa GBP and the Gastrin/CCK-C Receptor

The IC_{50} values determined for inhibition of cross-linking of ^{125}I-[Nle^{15}]-$gastrin_{2,17}$ to the 78 kDa GBP by $gastrin_{17}$, CCK_8 and their analogues correlate well with the values previously reported for the gastrin/CCK-C receptor on gastrointestinal cell lines (2,3), as shown in Figure 3B. In contrast, the correlation with the values reported for binding to the CCK-A and gastrin/CCK-B receptors (Figure 3A) is poor. Similarly, for the antagonists proglumide and benzotript a good correlation is observed in the case of the gastrin/CCK-C receptor only (Figure 3B).

Anti-proliferative Gastrin/CCK Receptor Antagonists Target the Gastrin/CCK-C Receptor

A good correlation is also observed between the IC_{50} values for inhibition of cross-linking of ^{125}I-[Nle^{15}]-$gastrin_{2,17}$ to the 78 kDa GBP by the non-selective gastrin/CCK receptor antagonists proglumide and benzotript, and the IC_{50} values for inhibition of HCT 116 colon carcinoma cell proliferation by the same antagonists (2; Table 1). We have previously reported that

the CCK-A receptor-selective antagonist L364,718 and the gastrin/CCK-B receptor-selective antagonist L365,260 had no effect on colon carcinoma cell proliferation at concentrations as high as 1 μM. Furthermore, the inhibition observed at higher concentrations of L364,718 was not reversed by addition of $gastrin_{17}$ or CCK_8SO_4, and hence was not caused by binding to cell surface gastrin or CCK receptors (4).

We have now found that neither L364,718 nor L365,260 has any effect on cross-linking of ^{125}I-[Nle^{15}]-$gastrin_{2,17}$ to the 78 kDa GBP at concentrations up to 200 μM. The effects of antagonists on cross-linking are consistent with the conclusion that binding of antagonists to the gastrin/CCK-C receptor inhibits cell growth.

NSAIDS inhibit cell proliferation via the GBP

CyclooxygenaseS are involved in prostaglandin synthesis, and utilize fatty acid derivatives as substrates; they are widely thought to be the targets for NSAIDS. The rat mitochondrial trifunctional protein (MTP), the α-subunit of which has strong amino acid sequence homology to the porcine GBP, also utilizes fatty acid derivatives as substrates. We have shown that NSAIDS, with the exception of acetaminophen, inhibit cross-linking of iodinated gastrin to the porcine GBP, and conclude that GBP may be the target of the anti-proliferative effect of these agents on colon cancer.

Hereditary defects in the MTP can cause death in early childhood (29). A wide range of diagnostic symptoms has been reported, including diarrhoea, vomiting, hypoketotic hypoglycaemic coma, myopathy and cardiomyopathy. Pathological examination reveals widespread fat deposition in liver, heart and kidneys. Similar symptoms and pathology have been described in Reye's syndrome, which affects children treated with aspirin during viral infection (30). We suggest that the

previously unsuspected link between these syndromes is the MTP. Limitation of caloric intake during illness would result in an increased reliance as a metabolic fuel on acetyl CoA, which is one product of the reaction catalysed by the β-subunit of the MTP. Blockade of fatty acid oxidation by the MTP, either by mutation or by inhibition with aspirin, would then result in hypoketotic hypoglycemia. Hence the observation of a negative correlation between acetaminophen usage and Reye's syndrome is consistent with the failure of acetaminophen to inhibit cross-linking of gastrin to the GBP at concentrations as high as 10 mM. This hypothesis predicts that NSAIDS will not inhibit the proliferation of cell lines derived from patients with MTP mutations, and enables rational design of more potent and selective MTP/GBP antagonists for use in novel therapies for colon cancer.

Expression of gastrin CCK-C receptors by YAMC and LIM 1215 cells

We have previously reported that LIM 1215 colon carcinoma cells express cell-surface gastrin/CCK-C receptors (3). This observation was consistent with our observation that proliferation of LIM 1215 cells was inhibited by the non-selective gastrin/CCK receptor antagonist proglumide (Example 9, and our unpublished results 10), and with the lack of inhibition by the CCK-A receptor-selective antagonist L364,718 or the gastrin/CCK-C receptor-selective antagonist L365,260 at concentrations as high as 1 μM (4).

We have now found that the conditionally immortalised mouse colon cell line YAMC also expresses cell surface gastrin/CCK-C receptors, but not CCK-A or gastrin/CCK-B receptors. YAMC cell proliferation is unaffected by the selective antagonists L364,718 and L365,260 at concentrations as high as 0.1 μM, but is inhibited by the non-selective gastrin/CCK receptor antagonists proglumide and benzotript. Moreover the IC_{50}

values which we have observed for these antagonists are consistent with the hypothesis that the gastrin/CCK-C receptor is the target for the inhibitory effects (6).

Role of the Gastrin/CCK-C Receptor in Autocrine Growth

Without wishing to be bound by any proposed mechanism for the observed effect, our observations provide a possible explanation for the inhibitory effects of gastrin/CCK receptor antagonists on cell proliferation. Competition between antagonists and gastrin, and between acyl CoA derivatives and gastrin, in the GBP cross-linking assay implies that antagonists will compete for substrate binding to the 78 kDa GBP. If the GBP possesses enoyl CoA hydratase and 3-hydroxyacyl CoA dehydrogenase activities with longer chain acyl CoA derivatives as substrates, as suggested by the binding of short chain acyl CoA derivatives (Figure 1) and by sequence comparisons with other members of the hydratase/dehydrogenase family (7), then presumably inhibition of fatty acid metabolism by antagonist will block cell growth, because of a reduction either in energy supply or in the availability of essential lipids. Although the competition observed between gastrin and acyl CoA derivatives suggests that gastrin might also inhibit one or both activities, gastrin is able to reverse the inhibition of HCT 116 cell proliferation by proglumide (2), implying that gastrin itself does not block any essential function of the 78 kDa GBP. Direct measurements of the effect of gastrin on the enzyme activities of the purified 78 kDa GBP are required to resolve this inconsistency.

The inhibition of HCT 116 cell proliferation by proglumide and benzotript, and the reversal of inhibition by concentrations of gastrin in the mM range, is consistent with the 78 kDa GBP being a component of an extracellular autocrine loop involving gastrin. However, no such reversal of proglumide inhibition by gastrin is observed with LIM 1215 or LIM 2412 cells (10), although the observed

competition between antagonists and gastrin for the 78 kDa GBP implies that reversal should be observed if the GBP is accessible to exogenous gastrin. One possible explanation for this discrepancy is that the 78 kDa GBP, the mRNA for which is expressed in all colon carcinoma cell lines tested so far, may appear on the cell surface in only a subset of colon carcinoma cell lines. The observation that of 5 colon carcinoma cell lines tested only HCT 116 and LoVo cells secreted progastrin (1) is also consistent with the hypothesis that only a subset of cell lines utilise gastrin in an extracellular autocrine loop.

Inhibition of proliferation of both YAMC and LIM 1215 cells by expression of antisense gastrin mRNA provides clear evidence for the presence in both cell lines of an autocrine loop involving progastrin-derived peptides. Our evidence implicates the gastrin/CCK-C receptor in the autocrine loop in both YAMC and LIM 1215 cells. While binding studies clearly localise a population of the gastrin/CCK-C receptor to the cell surface in both YAMC and LIM 1215 cells (Table 3), subcellular fractionation has localised a second population of the gastrin/CCK-C receptor to mitochondrial membranes in rat liver (31). Furthermore the failure to reverse antisense inhibition by exogenous $gastrin_{17}$ indicates that the autocrine loop is exclusively intracellular. We therefore postulate that a novel intracellular autocrine (i.e. intracrine) loop involving amidated gastrin and the gastrin/CCK-C receptor contributes to proliferation of both YAMC and LIM 1215 cells.

It may therefore be necessary to screen biopsy samples for the presence of cell-surface GBP in order to determine the suitability of a cancer for treatment with an antagonist according to the method of the present invention. Such screening may be carried out using antibody to GBP, labelled with a detectable marker, and measuring the binding of the labelled antibody to dispersed tumour cells. Alternatively screening may be carried out

as described by Weinstock and Baldwin (3).

The use of immunochemical assays of inhibition of binding of labelled antibody, or assays of inhibition of binding of labelled high-affinity antagonists, provide the opportunity for automated assays which can be used for large scale screening of putative antagonists. Assays of the enzyme activities intrinsic to the GBP may also be used for large-scale screening of GBP antagonists. Covalent cross-linking of ^{125}I-gastrin$_{2,17}$ to the purified 78 kDa GBP provides a convenient assay with which to isolate more potent and selective GBP antagonists capable of blocking cell growth.

References cited herein are listed on the following pages.

It will be clearly understood that the invention in its general aspects is not limited to the specific details referred to herein.

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CLAIMS:

1. A method of identifying a compound having the ability to block the low-affinity gastrin-cholecystokinin type C receptor, comprising the step of measuring the ability of said compound to block the binding to gastrin-binding protein of a compound selected from the group consisting of a gastrin-related peptide, a cholecystokinin-related peptide, an antagonist of gastrin or of cholecystokinin, an acyl CoA, an enoyl CoA, an antibody to gastrin, and an antibody to cholecystokinin.

2. A method according to Claim 1 in which the binding is irreversible.

3. A method according to Claim 2 in which inhibition of irreversible binding is assessed by measuring the ability of the compound to block cross-linking of a gastrin analogue to gastrin binding protein.

4. A method according to Claim 3 in which the cross-linking is effected by a homobifunctional cross-linking agent.

5. A method according to Claim 4 in which the homobifunctional cross-linking agents is selected from the group consisting of disuccinimidyl suberate, disulfosuccinimidyl suberate, and ethylene glycol bis-(succinimidyl)-succinate.

6. A method according to Claim 3 in which the cross-linking is effected by a heterobifunctional cross-linking agent.

7. A method according to Claim 6 in which the heterobifunctional cross-linking agent is succinimidyl-4-(p-maleimidophenyl) butyrate.

8. A method according to Claim 3 in which the cross-linking is effected by a photoreactive cross-linking agent.

9. A method according to Claim 8 in which the photoreactive agent is N-hydroxysuccinimidyl-4-azidobenzoate.

10. A method according to any one of Claims 3 to 9 in which the detectable marker is biotin or is a radioactive

or fluorescent label.

11. A method according to Claim 1 in which the binding is reversible.

12. A method according to Claim 11 in which the ability of the putative antagonist to prevent binding of a known agonist or antagonist of very high affinity is measured, said known agonist or antagonist being labelled with a detectable marker.

13. A method according to Claim 12 in which the known agonist or antagonist has at least the binding affinity of benzotript.

14. A method according to Claim 12 or Claim 13 in which the detectable marker is ^{3}H.

15. A method according to Claim 11 in which the ability of the putative antagonist to prevent binding of a high affinity antibody is measured, said antibody being labelled with a detectable marker.

16. A method according to Claim 15, in which the antibody is capable of blocking binding of an acyl CoA or of gastrin to gastrin-binding protein.

17. A method according to Claim 16 in which the antibody is a monoclonal antibody.

18. A method according to Claim 1 in which the inhibition of the enoyl CoA hydratase and/or 3-hydroxyacyl CoA dehydrogenase activity intrinsic to the GBP is assayed.

19. A method according to Claim 18 in which the hydratase or dehydrogenase activity is measured spectrophotometrically or using a substrate labelled with a radioactive marker.

20. A method according to Claim 19 in which the activity is measured spectrophotometrically by the decrease in enoyl CoA absorption on hydration or by the decrease in NADH absorption on oxidation.

21. A method according to any one of Claims 1 to 20 in which the gastrin-related peptide is $[Leu^{15}]gastrin_{17}$ or $[Nle^{15}]gastrin_{2,17}$.

22. A method according to Claim 21 in which the gastrin-related peptide is [Nle^{15}]gastrin$_{2,17}$.

23. A pharmaceutical composition of a compound with the ability to inhibit the binding of a gastrin analogue to the low-affinity gastrin-cholecystokinin type C receptor, comprising a pharmaceutically-effective amount of said compound and a pharmaceutically-acceptable carrier, with the proviso that the compound is not idomethacin, sulindac, piroxicam, aspirin, proglumide or benzotript.

24. A composition according to Claim 23, in which the compound is a non-selective antagonist of the gastrin-cholecystokinin receptor.

25. A composition according to Claim 24 in which the antagonist has an IC_{50} value in the millimolar range or lower.

26. A composition according to any one of Claims 23 to 25 which additionally comprises another pharmaceutically-active molecule.

27. A composition according to Claim 26 in which the other pharmaceutically-active molecule is an antacid.

28. A composition according to Claim 26 in which the other pharmaceutically-active molecule is an anti-cancer agent.

29. A composition according to Claim 28 in which the anti-cancer agent is a cytokine.

30. A method of controlling gastric acid secretion, comprising the step of administering to a mammal in need of such treatment an effective amount of a compound having the ability to inhibit binding of a gastrin peptide to gastrin-binding protein.

31. A method according to Claim 30, comprising the step of administering a composition according to Claim 27.

32. A method of treating neoplastic disease comprising the step of administering to a mammal in need of such treatment an effective amount of a compound having the ability to inhibit binding of a gastrin peptide to gastrin-binding protein, with the proviso that the compound is not

indomethacin, sulindac, piroxicam, aspirin, proglumide or benzotript.

33. A method according to Claim 32, in which the neoplastic disease is a cancer of the gastrointestinal tract.

34. A method according to Claim 32, in which the neoplastic disease is selected from the group consisting of colon carcinoma, gastric carcinoma, mammary carcinoma, malignant melanoma, tumours of epithelial origin, and lymphomas.

35. A method according to any one of Claims 32 to 34, comprising the step of administering a composition according to Claim 28 or Claim 29.

36. A method according to any one of Claims 32 to 35, in which the compound having the ability to inhibit binding of the gastrin peptide to gastrin-binding protein is a non-steroidal anti-inflammatory compound.

37. A method according to Claim 36 in which the non-steroidal anti-inflammatory compound is an inhibitor of prostaglandin activity.

38. A method of treatment of a neoplastic disease, comprising the step of inducing production of antisense gastrin mRNA in neoplastic tissue of an animal in need of such treatment.

39. A method according to Claim 38, in which tissue-specific production of anti-sense mRNA is achieved by targeting of corresponding DNA to the desired tissue, using tissue-specific promoters.

40. A method according to Claim 38 or Claim 39, in which the neoplastic disease is selected from the group consisting of colon carcinoma, gastric carcinoma, other gastrointestinal cancers, mammary carcinoma, malignant melanoma, tumours of epithelial origin, and lymphomas.

FIG. 1A

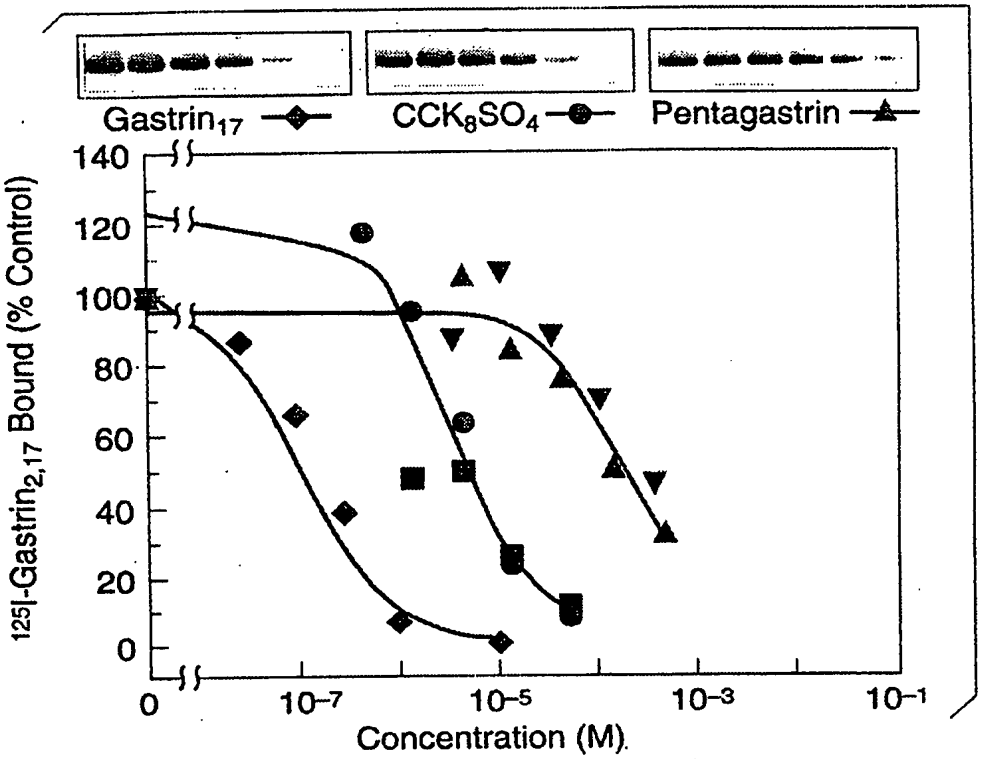

Gastrin17
CCK8SO4
Pentagastrin
140
120
100
80
60
40
20
0
125I-Gastrin2,17 Bound (% Control)
0
10-7
10-5
10-3
10-1
Concentration (M)

FIG. 1B

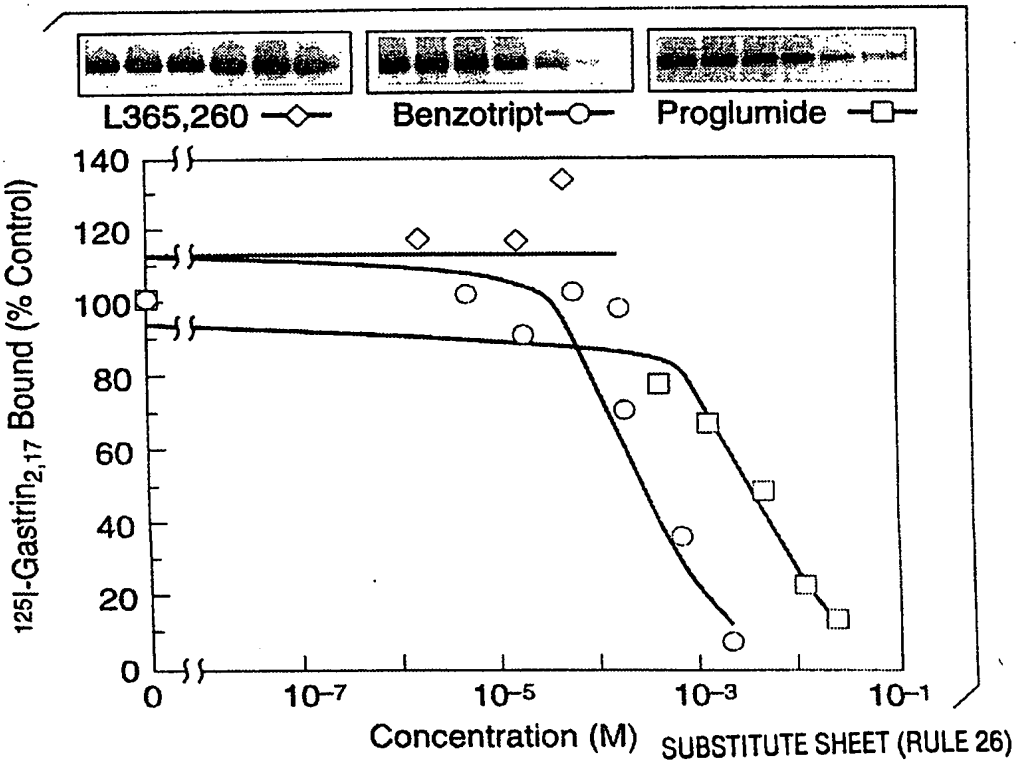

L365,260
Benzotript
Proglumide
140
120
100
80
60
40
20
0
125I-Gastrin2,17 Bound (% Control)
0
10-7
10-5
10-3
10-1
Concentration (M)

FIG. 2

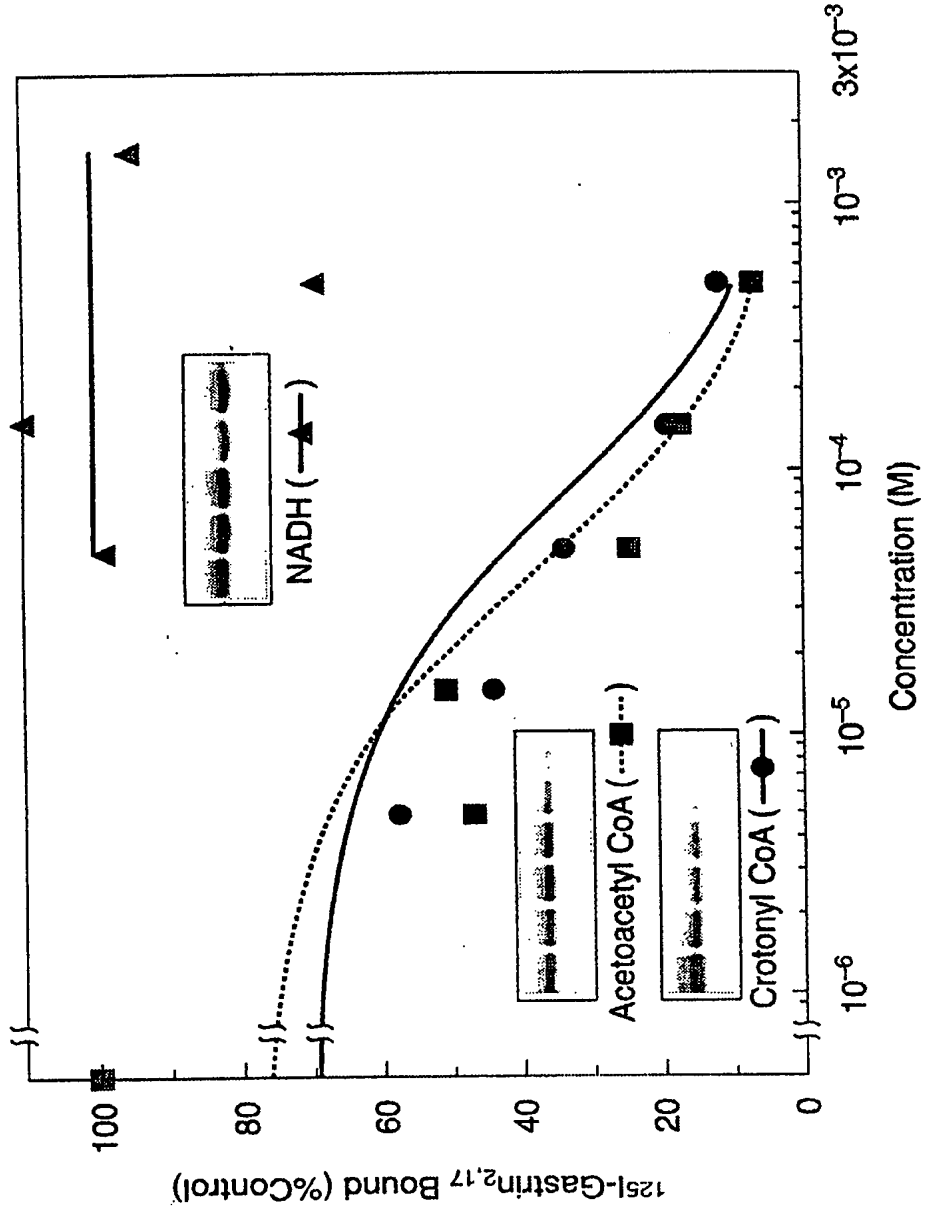

125I-Gastrin2-17 Bound (%Control)
100
80
60
40
20
0
Concentration (M)
10-6
10-5
10-4
10-3
3x10-3
NADH
Acetoacetyl CoA
Crotonyl CoA

FIG. 3A

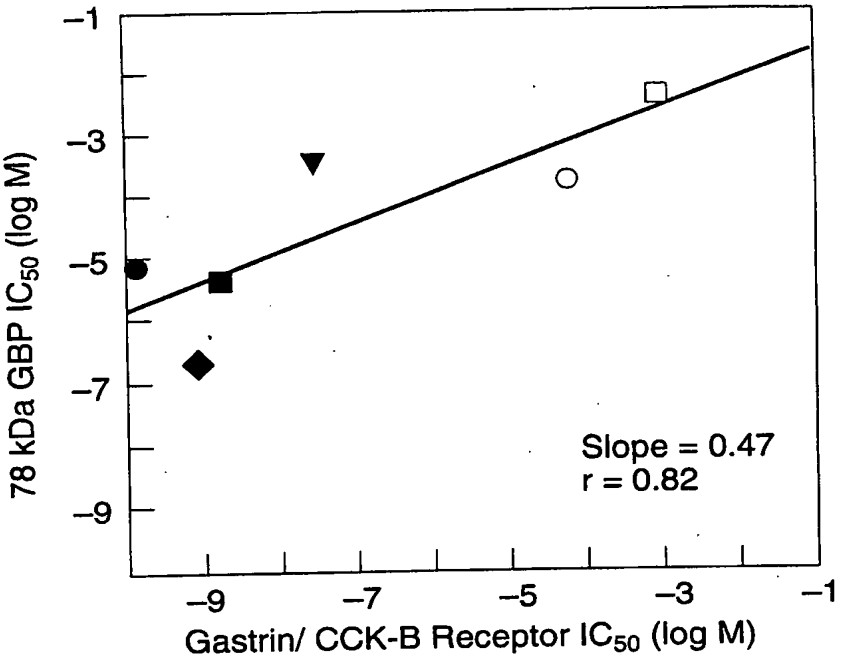
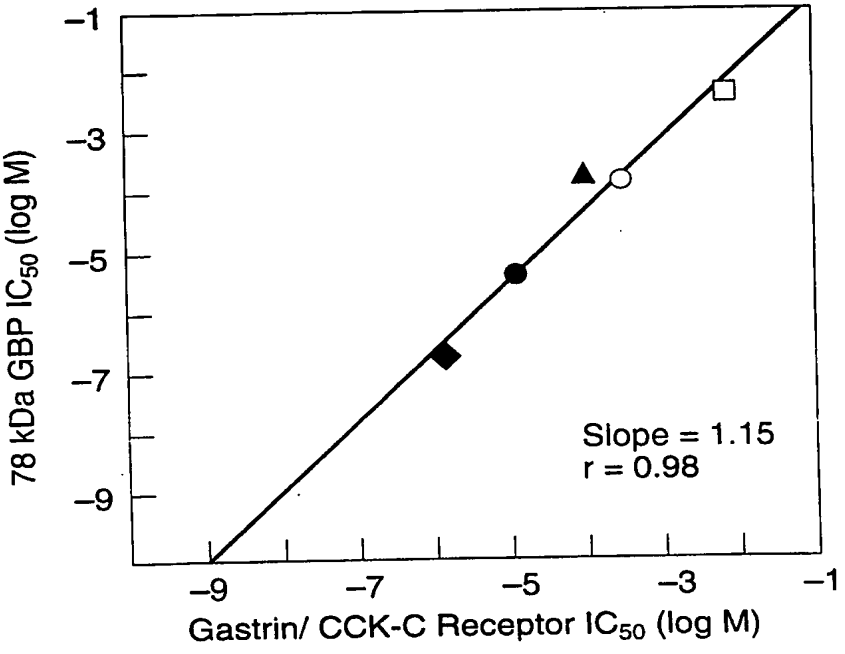

78 kDa GBP IC_{50} (log M)
−1
−3
−5
−7
−9
Slope = 0.47
r = 0.82
−9
−7
−5
−3
−1
Gastrin/ CCK-B Receptor IC_{50} (log M)

FIG. 3B


78 kDa GBP IC_{50} (log M)
−1
−3
−5
−7
−9
Slope = 1.15
r = 0.98
−9
−7
−5
−3
−1
Gastrin/ CCK-C Receptor IC_{50} (log M)

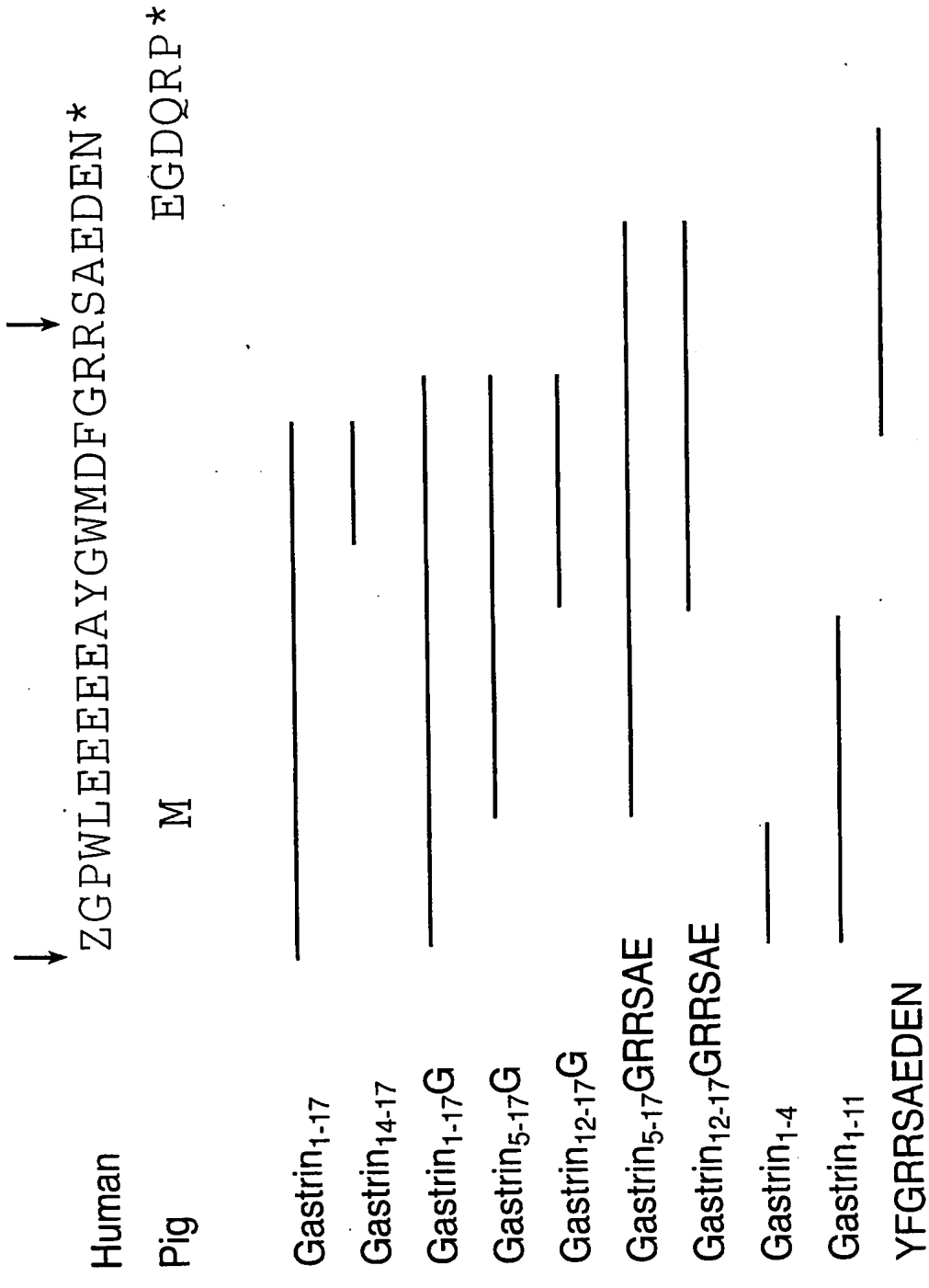

ZGPWLEEEEAYGWMDFGRRSAEDEN*
Human
M
Pig
EGDQRP*
Gastrin1-17
Gastrin14-17
Gastrin1-17G
Gastrin5-17G
Gastrin12-17G
Gastrin5-17GRRSAE
Gastrin12-17GRRSAE
Gastrin1-4
Gastrin1-11
YFGRRSAEDEN

FIG. 5A

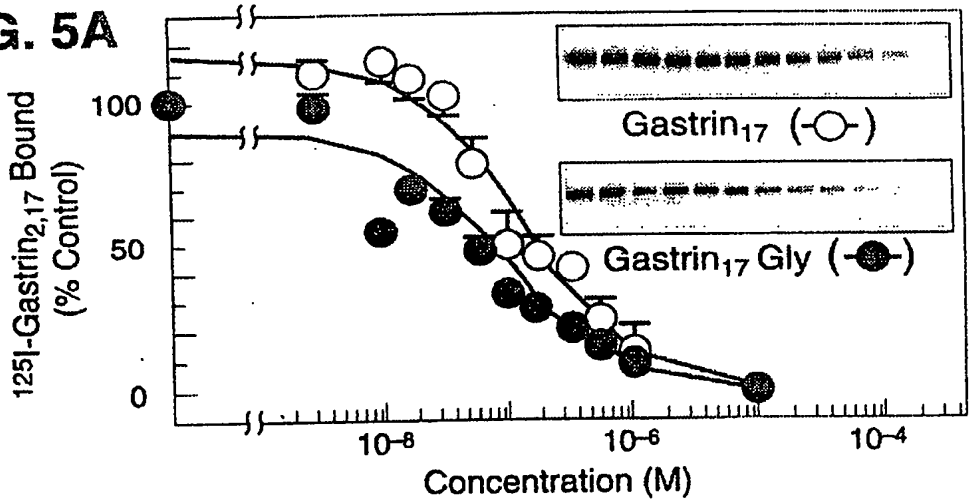


FIG. 5B

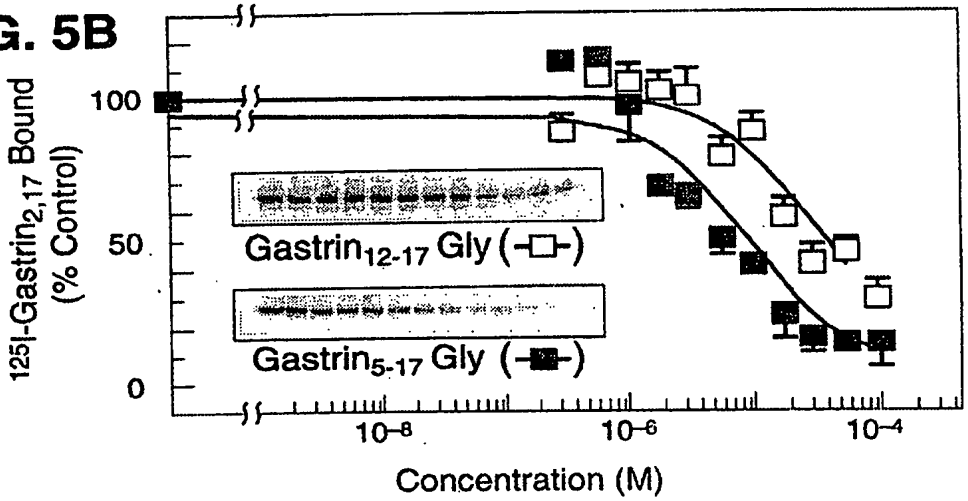


FIG. 5C

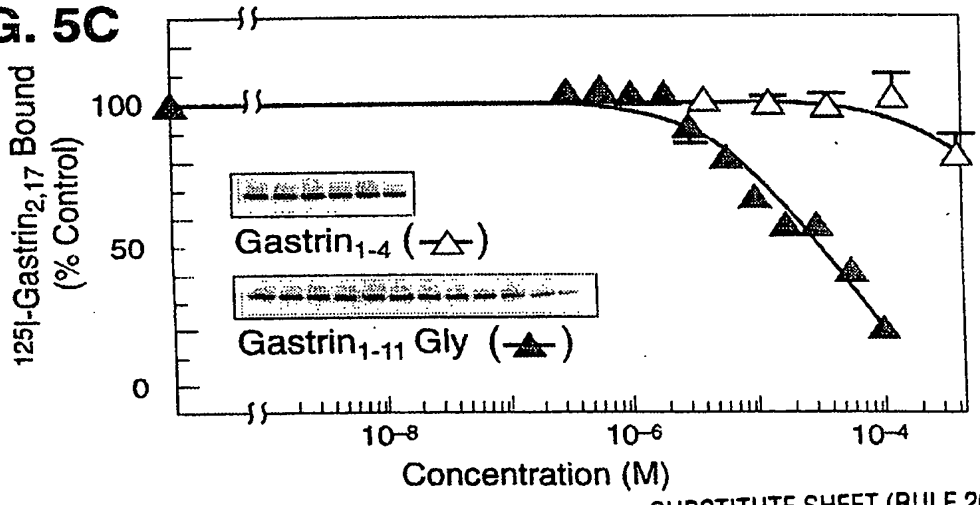


FIG. 6A

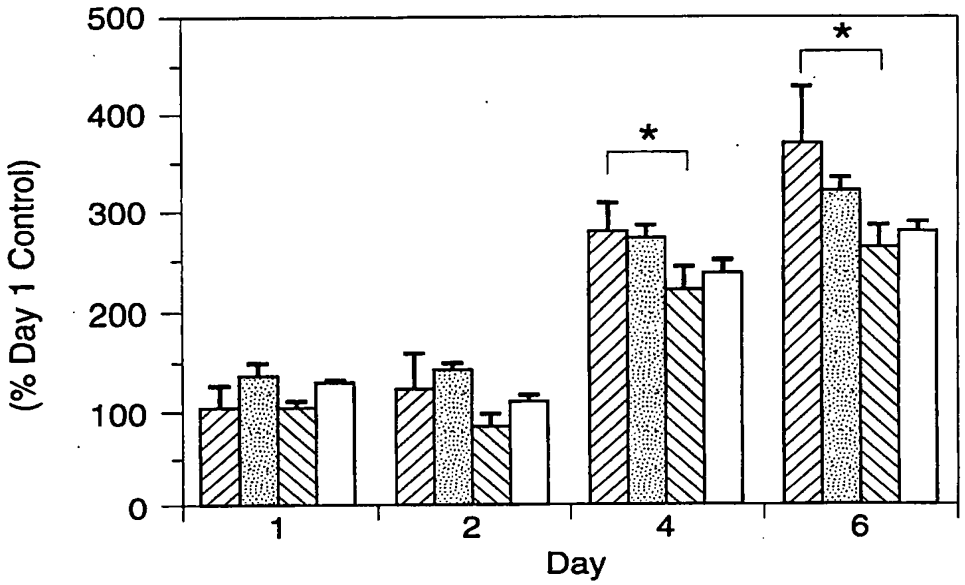

500
400
300
200
100
0
(% Day 1 Control)
*
*
1
2
4
6
Day

FIG. 6B

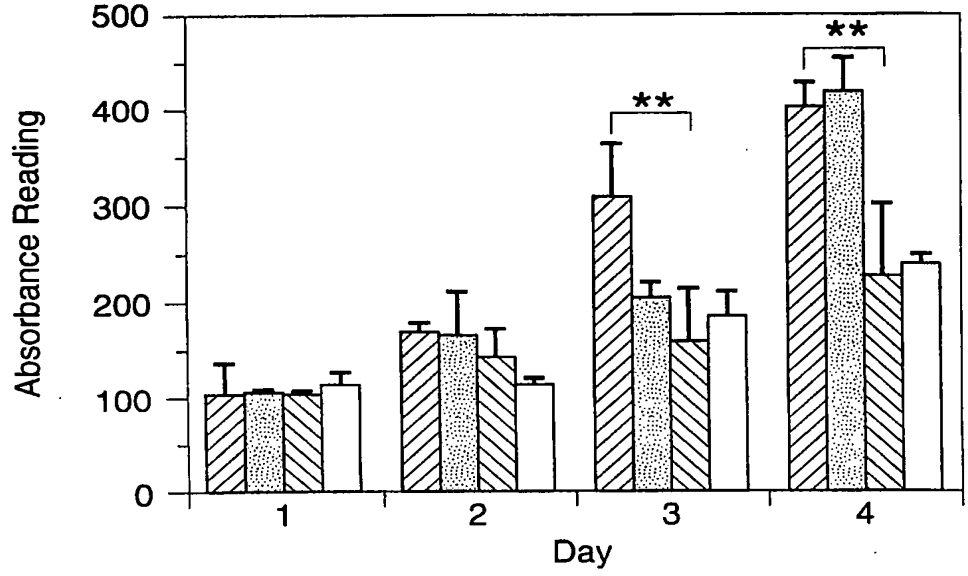

500
400
300
200
100
0
Absorbance Reading
**
**
1
2
3
4
Day

FIG. 7

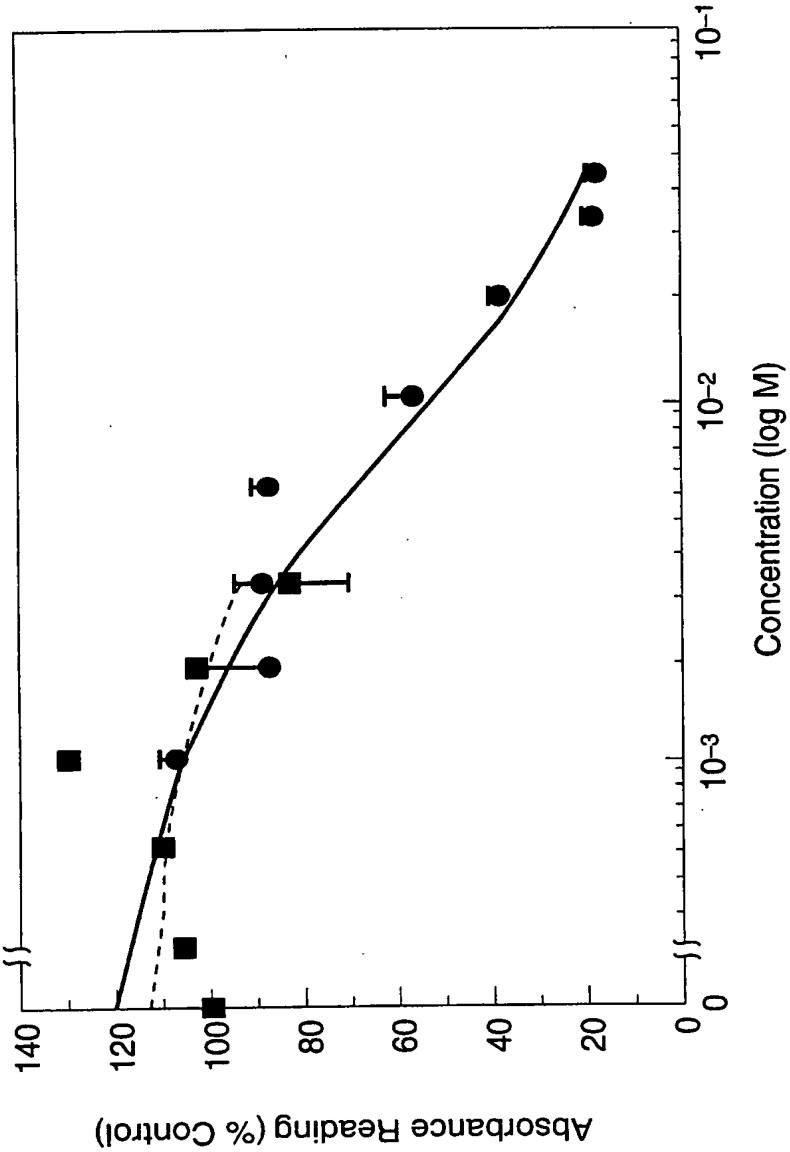

Absorbance Reading (% Control)
140
120
100
80
60
40
20
0
0
10-3
10-2
10-1
Concentration (log M)

FIG. 8A

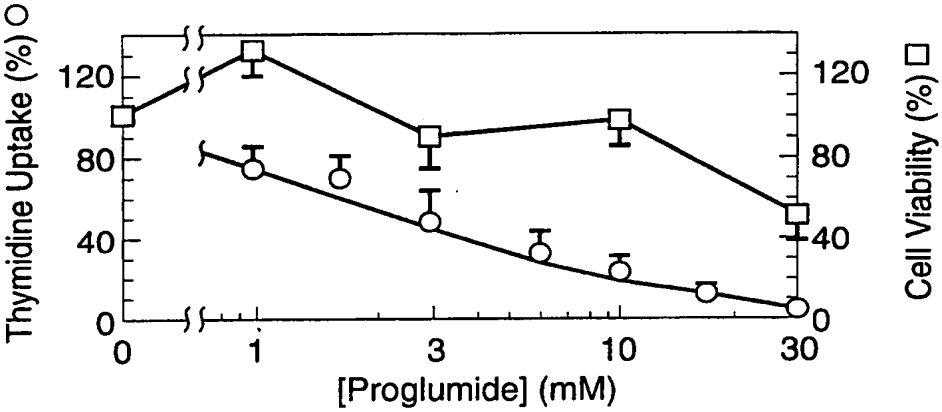


FIG. 8B

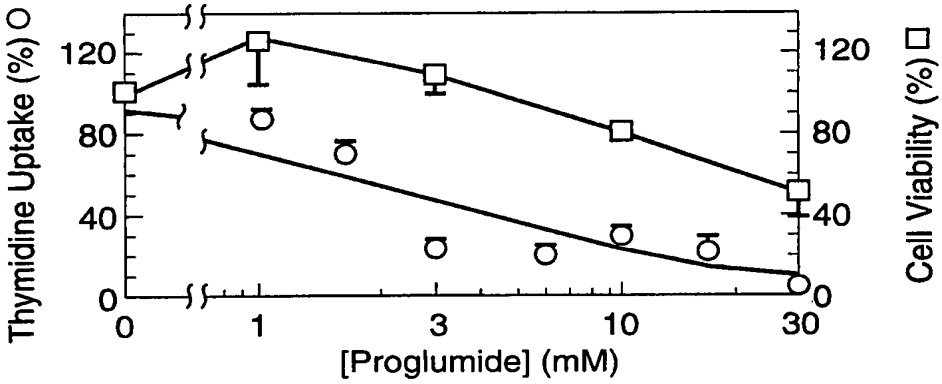
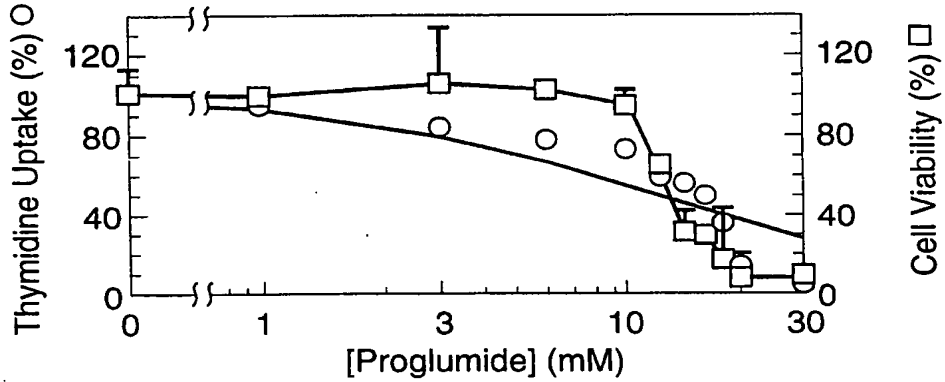


FIG. 8C



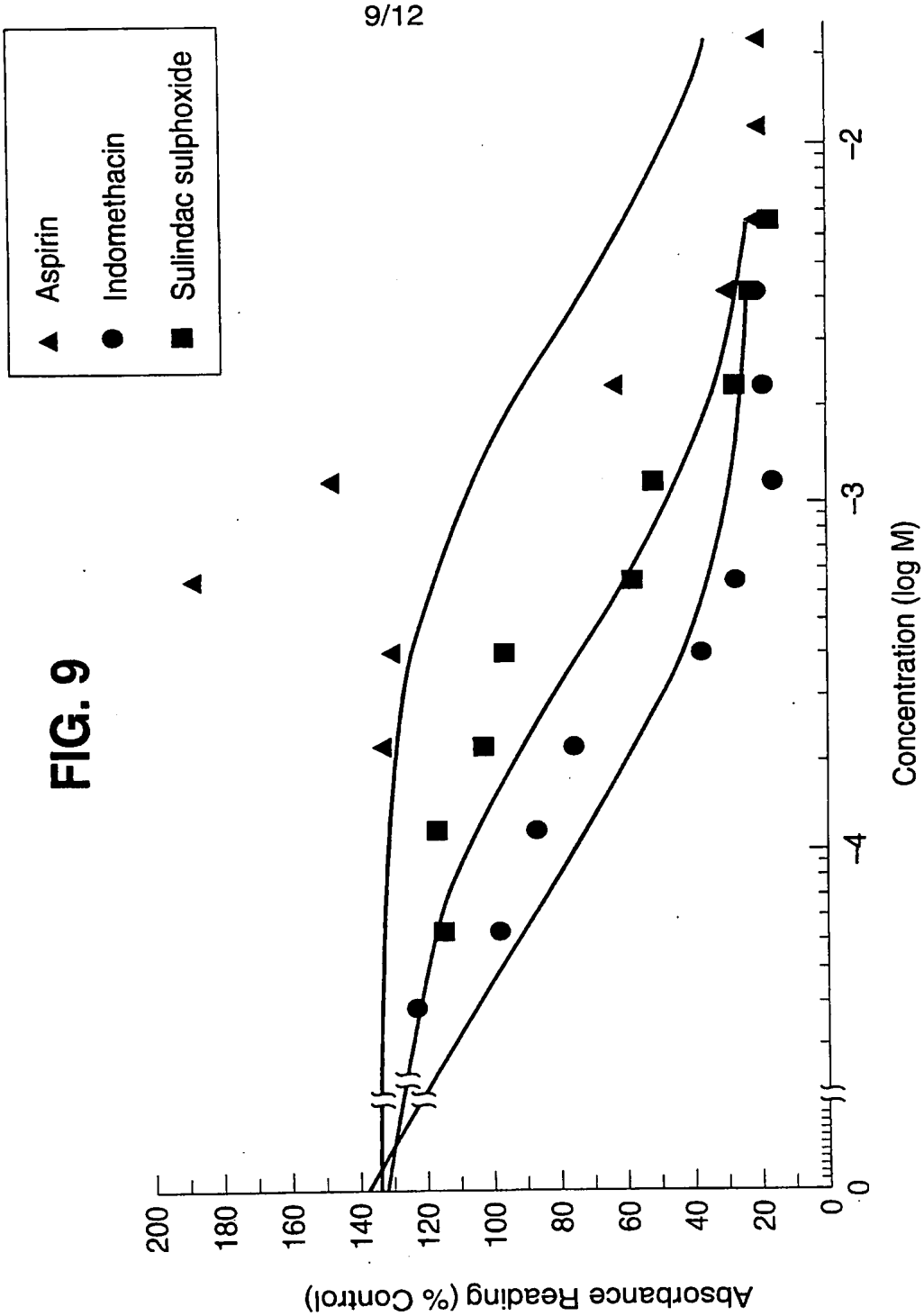

Aspirin
Indomethacin
Sulindac sulphoxide
Absorbance Reading (% Control)
200
180
160
140
120
100
80
60
40
20
0
Concentration (log M)
-2
-3
-4
0

FIG. 9

FIG. 10A

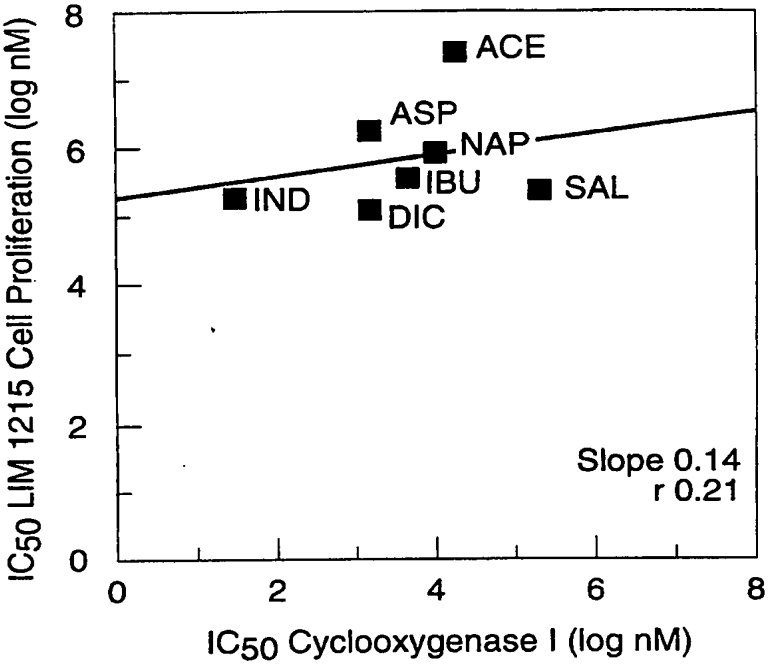


FIG. 10B

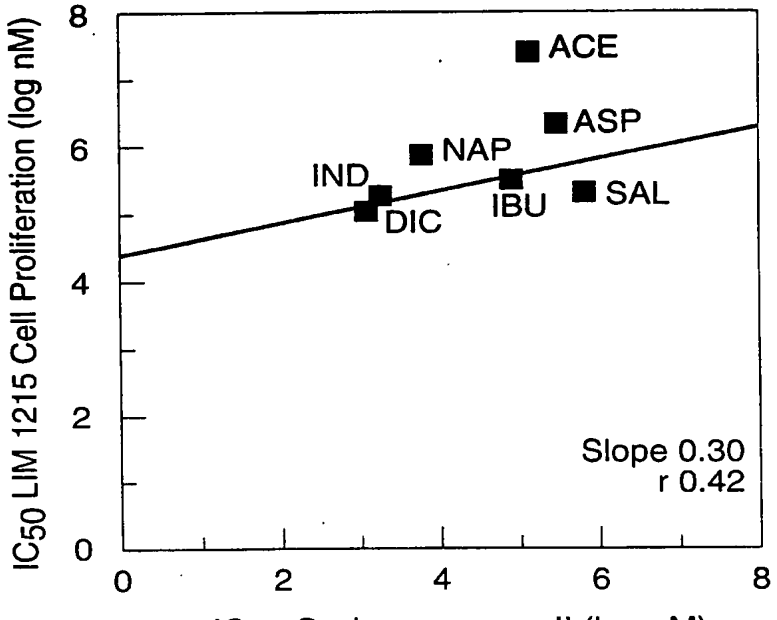
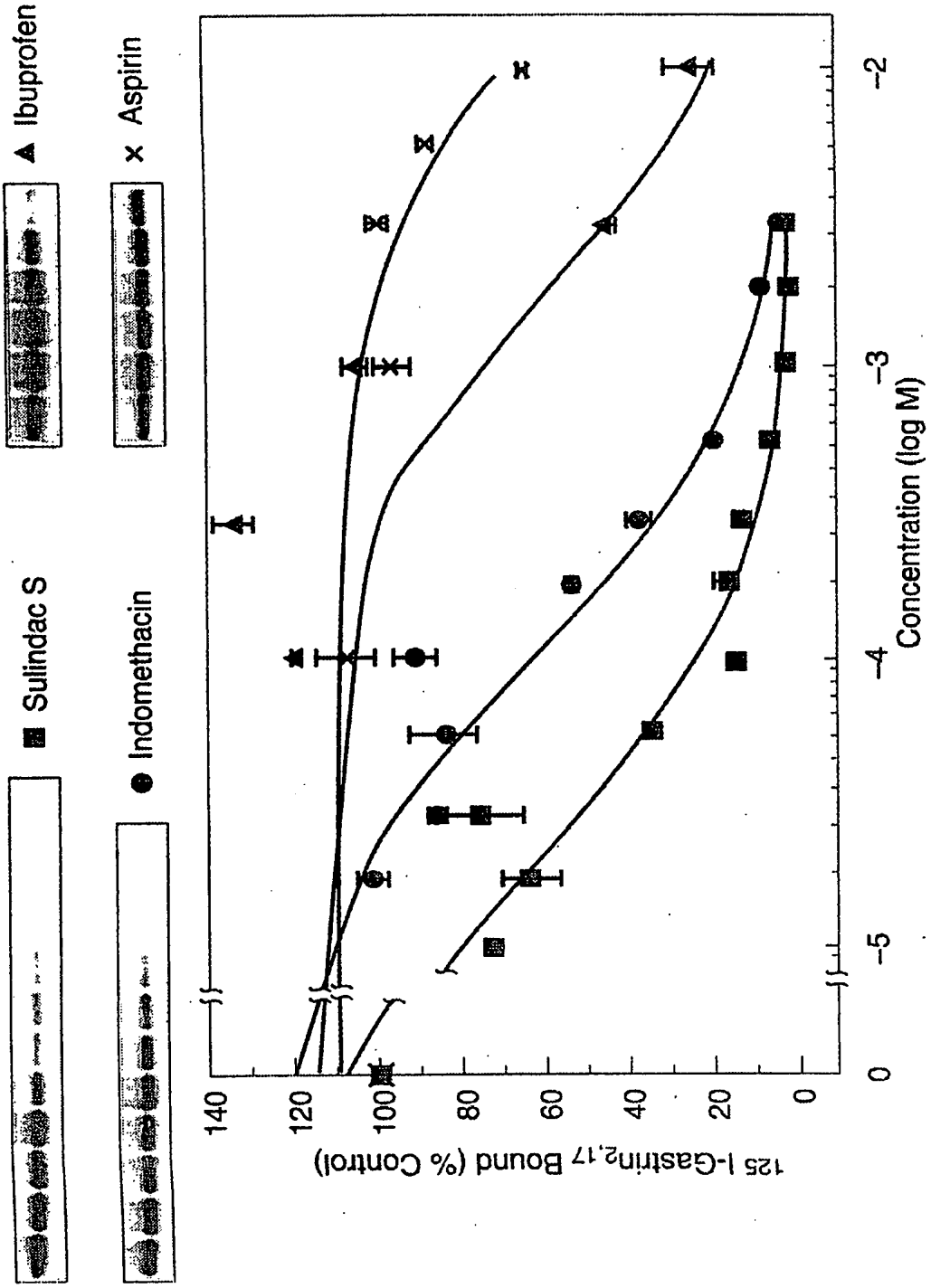


FIG. 11


▲ Ibuprofen
× Aspirin
■ Sulindac S
● Indomethacin
125 I-Gastrin2.17 Bound (% Control)
140
120
100
80
60
40
20
0
0
-5
-4
-3
-2
Concentration (log M)

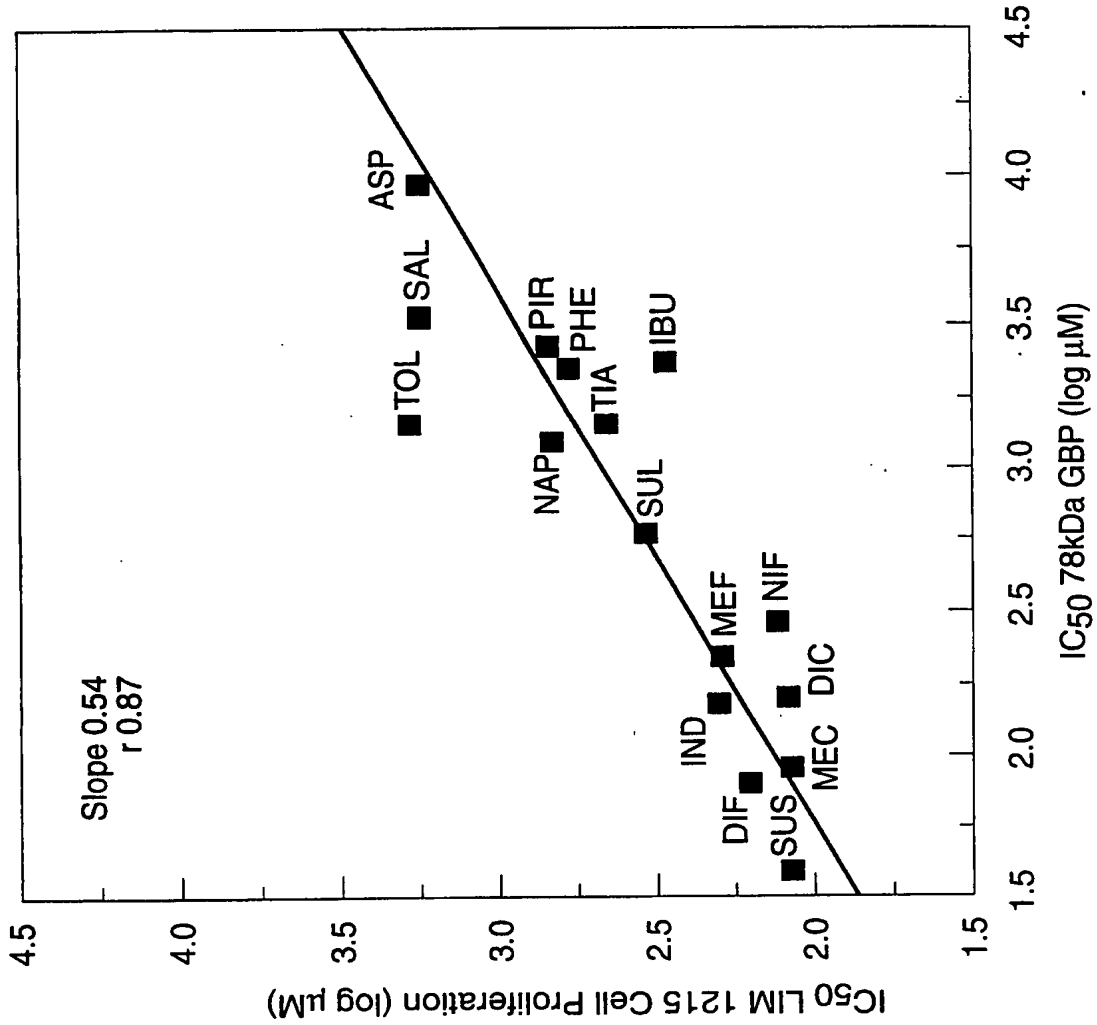

ASP
SAL
TOL
PIR
PHE
TIA
IBU
NAP
SUL
MEF
NIF
DIC
IND
MEC
DIF
SUS
Slope 0.54
r 0.87
IC50 LIM 1215 Cell Proliferation (log µM)
IC50 78kDa GBP (log µM)
4.5
4.0
3.5
3.0
2.5
2.0
1.5

FIG. 12

INTERNATIONAL SEARCH REPORT

Inte .ional application No.
PCT/US95/01375

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/53, 33/573; A61K 31/70, 38/16, 48/00
US CL :435/7.2, 7.4; 514/2, 44; 530/309; 935/62
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.2, 7.4; 514/2, 44; 530/309; 935/62

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EMBO Journal, Vol. 8, issued 1989, D. Becker et al., "Proliferation of human malignant melanomas is inhibited by antisense oligodeoxynucleotides targeted against basic fibroblast growth factor," pages 3685-3691, see entire document.	38-40
X,P --- Y,P	Proceedings of the National Academy of Sciences USA, Vol. 91, issued August 1994, G.S. Baldwin, "Antiproliferative gastrin/cholecystokinin receptor antagonists target the 78-kDa gastrin-binding protein," pages 7593-7597, see entire document.	1-20, 23-30, 32-34 --- 38-40

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 26 APRIL 1995	Date of mailing of the international search report 15 MAY 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DONNA C. WORTMAN, PH.D Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Inte ional application No.
PCT/US95/01375

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences USA, Vol. 84, issued May 1987, F-T. Mu et al., "Monoclonal antibody to the gastrin receptor on parietal cells recognizes a 78-kDa protein," pages 2698-2702, see entire document.	1-20, 23-30, 32-34, 38-40
Y	Biochimica et Biophysica Acta, Vol. 1170, issued 1993, T. Mantamadiotis et al., "Nucleotide sequence encoding a novel member of the hydratase/dehydrogenase family," pages 211-215, see entire document.	1-20, 23-30, 32-34, 38-40
Y	Comparative Biochemistry and Physiology-B Comparative Biochemistry, Vol. 104B, No. 1, issued 1993, G.S. Baldwin, "Comparison of sequences of the 78 kDa gastrin-binding protein and some enzymes involved in fatty acid oxidation," pages 55-61, see entire document.	1-20, 23-30, 32-34, 38-40

INTERNATIONAL SEARCH REPORT

Ir. .ational application No.
PCT/US95/01375

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 21, 22, 31, 35-37
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Inte. .ional application No.
PCT/US95/01375

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-20, 23-30 and 32-34, drawn to method of identifying a compound, a compound and method of treatment using a compound.

Group II, claims 38-40, drawn to method of treatment involving antisense mRNA.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Group II lacks the same or corresponding special technical features shared by the claims of Group I because the method of Group II is a distinctly different method from the method of Group I involving the production of antisense mRNA, which is not a feature of the claims of Group I. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.